Human histocompatibility testing by T cell-mediated lympholysis. A European CML-standard technique. Report from the Third European CML Workshop, Marseille 1979.

Goulmy, E.A.J.M.; Kristensen, T.; Mawas, C.; Schendel, D.; Wank, R.; Johnsen, H.E.; ... ; Pfeffer, P.

issue Antigens (1980), 16, 335–367

Human Histocompatibility Testing by T Cell-Mediated Lympholysis: A European Standard CML Technique

Report from the European CML Study Group

on

The Third European CML Workshop

Centre D'Immunologie
INSERM-CNRS
Marseille, December 1979

Participating laboratories

(1) Tissue Typing Laboratory (AR),
University Hospital,
DK-8000 Aarhus C,
Denmark.

Supported by the Danish Medical Research Council.

(2) Department of Immunohaematology and Blood Bank (LEI 1 and 2),
University Medical Center,
Leiden,
The Netherlands.

Supported in part by the National Institutes of Health; The Dutch Organization for Health Research (TNO); the Dutch Foundation for Medical Research (FUNGO), which is subsidized by the Dutch Organization for the Advancement of Pure Research (ZWO); and the J.A. Cohen Institute for Radiopathology and Radiation Protection (IRS).

(3) Centre d'Immunologie INSERM-CNRS (MA),
de Marseille-Luminy – Case 906,
F-13288 Marseille cédex 9,
France.

Supported by INSERM (CRL 79.5.118.1; AU 78.150) and DGRST (ACC.77.7.1370; 78.7.3102).

0001–2815/80/010335–33 $02.50/0

(4) Institute fo. Immunology (MU),
Schillerstrasse 42,
D-8000 Munich,
W Germany

Supported by Deutsche Forschungsgemeinschaft (SFB 37 B3B4)

(5) Tissue Typing Laboratory (OS),
University Hospital,
Oslo 1,
Norway

Supported by the Norwegian Research Council for Science and the Humanities

Participants

Els Blockland (LEI 1), Ben Bradley (LEI 1), Hans Bruning (LEI 2), Dominique Charmot (MA) Els Goulmy (LEI 1), Hans E Johnsen (AR), Marry Kardol (LEI 2), Tom Kristensen (AR and MA), Claude Mawas (MA), Jette Mossin (AR), Per Pfeffer (OS), Jan Van der Poel (LEI 2) Dolores Schendel (MU), Rudi Wank (MU), Peter Wendelboe Hansen (AR)

Present coordinator Tom Kristensen (AR and MA)
to whom all correspondence should be sent

Received for publication 9 June, accepted 9 June 1980

The HLA–A, B, C and DR antigenic determinants of the human major histocompatibility system have been defined by antibody mediated cytotoxicity assays using highly selected alloantisera obtained from multiparous women, immunized human volunteers, or allotransplanted patients Using many sera which have been collated through the international histocompatibility workshops (e g Histocompatibility Testing 1977) it has been possible to define 60 distinct specificities genetically coded by the HLA–A, B, C and DR loci

The concept of histocompatibility-typing with cells ("cellology") was first introduced by scientists working with the Mixed Lymphocyte Culture (MLC) technique (Bradley et al 1972, Mempel et al 1973) With this technique, the HLA–Dw determinants and the HLA–D locus were established (Histocompatibility Testing 1975)

Subsequently the use of *in vitr* generated cytotoxic T-lymphocytes f histocompatibility typing was propos and initiated by serveral, separate grou (e g Mawas et al 1973, 1974, Kristens et al 1974) Both products of the HLA– B, C (e g Grunnet et al 1975, 1976) a the D/DR loci (Albrechtsen et al 197 Feighery & Statsny 1979, Johnsen 198 may be recognized by cytotoxic lymph cytes, but it is not yet clear whether t actual determinants recognized by cyt toxic T lymphocytes are identical to the recognized by antibody, as had be assumed from early CML-studies (review by Eijsvoogel et al 1973) Thus, a colla orative effort to examine the relationsh between the determinants defined serology and cellology was initiated 1977 (Kristensen 1978) The fi investigations performed locally and t data obtained from CML-Workshop

(Goulmy et al 1976, 1977, Kristensen & Grunnet 1975, Kristensen et al 1976, Kristensen 1978, Schendel et al 1977, 1978a, Bradley et al 1978) suggested that cytotoxic T lymphocytes recognize antigen differently than do available, highly selected HLA antisera, or in fact may recognize products under different genetic control In order to investigate these models on an international basis, two further European CML-Workshops were initiated with the aim of standardizing the CML-technique before proper immunogenetic investigations were undertaken The results of CML-Workshop II have been published previously (Bradley et al 1980) This report, covering CML-Workshop III, presents the *European Standardized CML-technique for histocompatibility typing* It contains detailed technical descriptions as well as procedures for data-analysis and criteria for assignment of positive and negative responses

Recently it has become possible to propagate and clone cytotoxic T-cells (Bonnard et al 1978, Schendel et al 1978b, 1980, Charmot et al 1980, Malissen et al 1979, 1980, Goulmy et al 1980) These techniques — much like monoclonal antibodies — may allow fine structural analysis of target cell properties both on genetic and biochemical levels, adding to our understanding of the HLA-system Consequently, aspects of these techniques were included in Workshop III and will be employed in future CML-Workshops

Goals of Workshop III

Based on the results of European CML-Workshop II (Bradley et al 1980) a partially standardized protocol was developed, utilizing not only similar procedures but also common sources of basic reagents The purpose in establishing such a protocol for Workshop III was to test (1) the same genetic combinations with common reagents and techniques to assess if more uniform results among the participating laboratories could be obtained, (2) to compare this standard technique with two variations, i e generation of CTL in microtiter plates with direct addition of target cells and carboxyfluorescein-diacetate labeling of target cells, (3) to compare various methods of data analysis, (4) to test various CTL expansion techniques, including, specific restimulation in 2° MLC, restimulation with pokeweed mitogen (PWM), and expansion with conditioned medium containing TCGF (T-cell growth factor)

Methods

1 — The Standardized European CML (SE CML) technique

A detailed account of the technique is given in Table 1, which also includes indications of minor discrepancies performed according to local beliefs or necessities

The standard technique was utilized by the Aarhus, Leiden-1, Marseille and Munich laboratories

2 — Generation of CTLs in microtiter plates with direct addition of target cells

This technique, performed by Oslo, has been published in detail elsewhere (Hirschberg et al 1977)

Briefly, MLC combinations were established in triplicates in round-bottomed microculture plates on day zero of culturing A four fold titration of effector cells was used ($1 \times 10^5 - 1\ 25 \times 10^4$ cells/well), number of stimulator cells always being half of the number of effectors

Table 1
Luropean standardized CML technique

SEPARATION OF PBL (Aarhus)		Comments
Anticoagulant	Heparin Sodim Novo without preservative final concentrations 25 IU/ml	defimbrated blood (OS)
Dilution of blood	10 ml blood diluted with 10 ml TC-199 are divided between two glass tubes (100 X 15/17 mm)	Approx 70 ml diluted blood over 25 ml Ficoll (MA)
gradient solution	About 4 ml lymphoprep (Nygaard & Co, Oslo) are, through a long needle, placed below the diluted blood	
temp	All reagents, except the blood, are at 4°C before gradient centrifugation at room temperature	Room temperature for all reagents (MA)
speed	1,000 *g*	
time	20 min	
washing procedure	After harvest of MNC from 10 ml blood into a conical 10 ml Plastic tube (NUNC N° 1060), the cells are washed X 2 (500 *g* 10 min) in 10 ml TC-199 with 40 IU heparin per ml added	
FREEZING PROCEDURE	10×10^6 MNC are pelleted and resuspended in 0 5 ml (4°C) cooled serum pool Just before freezing 0 5 ml of a cooled mixture of 20% DMSO (Merck, cat n° 2950) in RPMI is added by pipette a little faster than "drop by drop" After gentle but quick resuspension, the 1 ml suspension is placed into polypropylene tubes (Cat n° 1076, Nunc, Roskilde, DK) These tubes are immediately placed in an uncovered rack in a − 80°C deepfreezer After 1 h or for convenience next day, the tubes are transferred to the final storage in the vapour phase of a nitrogen freezer (− 190°C) (Ref Jørgensen & Lamm 1974)	Freezing is done in medium + 20% plasma or serum + 10% DMSO (OS-Ma) and in one step cells are resuspended in freezing medium and distributed in 1 ml ampoules (Ma)
PREPARATION OF SERUM POOL (Aarhus)	4 male donors, blood group AB, donated 500 ml whole blood Immediately after coagulation at 4°C the bottles were centrifuged, and serum harvested and pooled, prior to	Plasma from heparinized blood from ~ 10 male donors may be used after inactivation at 56°C

Table 1 (contd)

PREPARATION OF SERUM POOL (Aarhus)		Comments
	separation into six aliquots of 200 ml The serum was inactivated at 56°C for 30 min and kept frozen at — 20°C	
SHIPPING PROCEDURES (Aarhus)	The frozen tubes were placed in ~ 12 kg of dry ice Packing was performed immediately before shipments The cells were planned to arrive in the local center during the next 12–36 h	
THAWING TECHNIQUE (Aarhus)	The tubes are placed in a test tube rack, and thawing is performed rapidly in a 37°C waterbath, when the last ice crystal is observed the suspension is transferred to a conical plastic tube (NUNC 1030) containing 5 ml cooled TC-199 (4°C), and the cells are immediately centrifuged at 200 *g* for 10 min This is followed by one wash in 5 ml TC-199 with spinning at 200 *g* for 5 min (room temperature) After resuspension in 25% SeRPMI the cells are ready for use	Ma uses TC-199 plus heparin to prevent cell aggregation and 0s uses DNAse for same purpose Only one wash for Ma, Lei 1, Lei 2, Ma, Lei 1, Lei 2 and 0s dilute the contents of ampules slowly to prevent osmotic shock
CULTURES FOR THE STIMULATION OF 1° CTL		
medium	RPMI 1640 (Gibco) with, L-glutamine, Penicillin 100 U/ml, Streptomycin 100 μg/ml 20% pooled serum from standardized source	
Culture vessel	50 ml culture flask/standing upright	Round bottomed culture plates — On day 0 cultures are set up in triplicates, with varying amounts of responder and stimulator cells (0s) — For few cells $\simeq 2 \times 10^6$, cultures in glass tubes (Ar) or in 2 ml Cluster plates (Ma)
volume	up to 20 ml flask, when volume is less than 5 ml the cells are cultured in Costar marco-well plates with 2 ml volume	

Table 1 (Contd.)

CULTURES FOR THE STIMULATION OF 1° CTL		Comments
cell concentration	Responding cells : 1×10^6/ml Stimulating cells : 1×10^6/ml	
culture ratios	1 RC : 1 SC_x	
inactivation of SC	2000 rads, x-irradiation	γ-irradiation for Ar, Lei 1, Lei 2, Ma, Os.
incubation conditions	38°C, 6% CO_2, well-humidified incubator, minimal opening of incubator during the culture time.	37°C, 5% CO_2 for Ar, Lei 1, Lei 2, Ma, Os.
culture duration	144 h.	
CULTURES FOR THE PRIMING OF CTL FOR USE IN 2° CML ASSAYS	The same circumstances as for the generation of primary CTLs, but the time of culture was 10 days.	No need for changing medium (Ma).
FREEZING PROTOCOL FOR PRIMED CTL	At day 10 a minimum of five 20 ml identical cultures were pooled and frozen in a number of $8-10 \times 10^6$ cells per tube *(c.f. freezing procedure, vide supra).*	
CULTURES FOR THE RESTIMULATION OF SPECIFIC 2° CTL		
medium	same as for 1° CTL	
culture vessel	*Cluster macro-well plates with 2 ml capacity*	Alternative choices: – 50 ml culture flasks standing upright (Ar – Ma). – round bottomed microplates (Os).
volume	1–2 ml/well	14 to 20 ml of medium in flasks (Ar.Ma).
cell concentration	*Responding cells : 1×10^6/ml* Stimulating cells : 1×10^6/ml	

Table 1 (Contd.)

CULTURES FOR THE RESTIMULATION OF SPECIFIC 2° CTL		Comments
inactivation of SC	2000 rads for 1° cultures	γ-irradiation (Ar. Lei 1, Lei 2, Ma, 0s).
incubation conditions	same as for 1° cultures	
culture duration.	120 h	
CULTURES FOR RESTIMULATION OF CTL WITH POKEWEED MITOGEN		
medium	RPMI 1640 + Antibiotics + 10% pooled plasma	25% pooled serum (Ar).
culture vessel	50 ml culture flasks – Standing upright	Glass tubes (Ar).
volume	10 to 20 ml	5 ml (Ar).
cell concentration	1×10^6 ml	3×10^6 C/5 ml (Ar).
PWM source	GIBCO	
preparation	Lyophylised – reconstituted in 10 ml sterile distilled water – no filtration – store at 4°C.	
concentration	1% final	
feeder layer	Autologous to the responding cells – 2×10^6 cells – irradiated 4000 rads.	
incubation conditions	37°C – 5% CO_2 – well-humidified incubator.	
culture duration	96 to 120 h	
additional comments	Feeder layer is not absolutely necessary but cell yields are much higher when adding this feeder layer.	

Table 1 (Contd)

CULTURES FOR PHA STIMULATION OF CML TARGET CELLS		Comments
medium	same as above	
culture vessel	50 ml culture flasks/standing upright	15 ml tubes, 4 ml total volume, 5×10^5 cells/ml for 0s
volume	5–10 ml	
cell concentration	1×10^6/ml	
PHA source	Difco PHA-m 0528-57	
preparation	Stock suspended in 5 ml RPMI, dilution of this 5 ml with 45 ml medium, filtration through 0 2 μ storage at – 20°C	
culture concentration	1 ml of the diluted PHA to 9 ml of medium = 1% of original stock stock	
incubation	same as above	
culture duration	72 h	After 56 h, 8 ml supernate is replaced by 8 ml fresh medium, to prevent aggregation of targets at the day of the assay (Lei 2).
CML ASSAY		
Preparation of CTL harvesting from flasks	Flasks are gently shaken – cells are transferred to conical 50 ml glass centrifuge tubes, tubes are centrifuged at 350 × *g* for 8 min and resuspended in fresh culture medium (2 ml combination)	
medium	Same as for 1° and 2° cultures	

Table 1 (Contd)

CML ASSAY *Preparation of CTL*		Comments
counting procedure	100 µl of the cell suspension added to 100 µl of trypan blue Live dead cell ratio determined cell concentration determined on basis of living cells only	
assay plate	V bottomed microtiter plates	Microtest plates (Terasaki type) (Lei 2)
distribution of CTL	100 µl of the starting suspension distributed to the plates, allowing duplicate well for each target (Note 5 target cells × 2 replicates × 100 µl per replicate = 1 ml of CTL suspension)	10 µl of starting suspension are distributed to the plates allowing triplicate wells for each target with Hamilton syringe A drop is formed at the needle tip and applied by touching the edge of the well Controls are heat treated effector cells incubated 15 min in 46° C water bath Controls are handled as above (Lei 2)
	After the first concentration is distributed 1 ml of culture medium is added to the remaining cell suspension giving a new volume of 2 ml The distribution is repeated as above A series of six dilutions is distributed to the plates with this sequential distribution and dilution pattern	Volume is 200 µl (Lei 2)
incubation	As soon as the CTL have been distributed to the plates they are immediately placed in an incubator until the target cells can be added	The CTLs are placed at room temperature until the targets are added (Ar)
		After distribution of the CTLs the trays are spun at 150 × *g* for 2 min and read in the automatic fluorometer (Lei 2)
Preparation of target cells		
harvesting	Flasks are gently shaken and cells transfered to 15 ml round bottomed plastic centrifuge tubes which are centrifuged at 350 × *g* for 8 min	
resuspension	Supernatant fluid is decanted and a small volume of about 200 µl is allowed to run back into the tube cell pellet is broken up and resuspended in this volume by scraping the tube across the top of a test tube rack	

Table 1 (Contd)

Preparation of target cells		Comments
labeling	250 μCi of $Na_2 Cr^{51} 04$ in a volume of 100 μl is added to each target sample	– 18 h before harvesting target cells (Os) – Targets are washed twice with Hanks The cells are labeled by resuspending in 1 ml of a fresh carboxyfluoresceindi-acetate solution in RPMI (final concentration 40 μg/ml) Lei 2)
incubation	Tubes are placed in a beaker containing water with a temperature of 38°C and placed in a 38°C, 6% CO_2 incubator	Water bath 37°C (Ar, Lei 1, Lei 2, Ma)
incubation time	1 h and 30 min, with shaking at 30 min intervals	15 min with shaking after 7 min (Lei 2)
washing procedures	3 ml of RPMI with antibiotics and 5% pooled serum is added to each tube This is then layered over a tube containing 5 ml of Lymphoprep ficoll solution This gradient is centrifuged at 400 g for 20 min The interface layer is removed and resuspended in 5 ml of the washing medium Cells are centrifuged at 350 × g for 8 min This washing is perfored 2 times	– No lymphoprep gradient, but 2 to 3 washes with medium (without plasma or serum) (Os Ma) – 10 ml of RPMI is added and the cells are centrifuged at 350 × g for 10 min After decantation of the supernate the targets are resuspended in 1 ml RPMI (without serum) (Lei 2)
distribution	Cells are counted as for CTL and suspended at 1 × 10^5/ml, 100 μl of this suspension is added to each well of the microtiter plate	Viability counting is done as for CTL and cells are suspended at 8 × 10^5/ml 5 μl of this suspension is added to wells of the microtest plate (Terasaki type) The tray is spun at 350 × g for 5 min after which the wells are flooded with medium After decanting the medium, effectors are added (Lei 2)
	Plates containing both CTL, and target cells are incubated for 4 h, incubation conditions identical to those described	Before incubation the plates are centrifuged 50 g, 5 min (Ar, Ma), 150 g,

Table 1 (Contd.)

CML-ASSAY-CONTINUED		Comments
harvesting of supernatants	100 μl aliquots are removed from each well after the plates are centrifuged at 450 × *g* for 5 min.	After incubation the trays are centrifuged at 450 × *g* for 5 min and flooded with phosphate buffer saline. After gentle shaking to wash away released carboxy-fluorescein the saline is decanted and the trays are read in the automatic fluorometer (Lei 2).
counting	Samples are counted for 1 min in a Nuclear Chicago Counter.	Commericial filter system harvester (Os, Lei 1).
Spontaneous release	Target cells are incubated in the microtiter plaes in wells containing only culture medium containing 20% serum with no CTL.	
maximum release	1 ml of the final target cell suspension is added to a centrifuge tube containing 1 ml of a 5% Triton-X detergent solution (New England Nuclear). These tubes are centrifuged at 450 × *g* for 10 min and 100 μl aliquots (x3) are counted.	Zaponine detergent may be used. It makes no difference with Triton × (Lei 1, Ma).
additional comments		The automated fluorometer (Lei 2). Inverted epi-illuminated fluorescent microscope (Leitz) equipped with an automated stage driven by stepping motor (Leitz) and a photomultiplier (Zeiss) encased in a black plastic box with counter balanced door. Digital equipment Corporation PDP 8/E computer (4K memory), high speed paper tape reader and puncher and a teleprint 303 writer. Light source is 60 W tungesten lamp mounted outside microscope box and fed from a stabilized power supply. Filters: standard FITc filter mirror combination with 6 mm BG 38 filters in excitation light path. Objective is a 6.3 × Neoflur.

Table 1 (Contd)

DATA ANALYSIS		Comments
raw data	cpm of duplicates recorded	Fluorometer readings before and after incubation are recorded The value of each individual well (i e = percentage fluorescence) in the final reading is divided by the value of the pre-reading in the corresponding well This ratio expressed as a percentage is used for calculation of % CML (Lei 2)
utilization of dilution data		
linear regression analysis r values slope determination		
calculation of % CML	$\frac{\text{mean ER- mean SR}}{\text{mean MR- mean SR}}$	$\left(1 - \frac{\text{mean test}}{\text{mean control}}\right) \times 100$ (Lei 2)
lytic unit size		
lytic units recovered/culture		
assignment of responses	r^2 greater than or equal to 0 90 (based on regression analysis) From this line the value of greater than or equal to 5% CML at a CTL Target Cell of 30 1 (Mu)	From dose response curves starting with an effector target ratio 50 100 1 Positive A minimum of two observations on the curve above 10% Negative All observations on the curve are less than 10% Intermediary Curves with only one observation above 10% (Ar) Specific ^{51}Cr release percentage $\leqslant$ 10% = neg, 11–15% weakly pos , 16–40% pos $\geqslant$ 41% strongly pos (Lei 1) Specific release percentage $\leqslant$ 10% = neg , 11–15% weakly pos 16–40% pos

After a 6 day sensitization period, the supertant in each well was aspirated, with a specially designed aspiration unit (Skatron A/S, Lierbyen, Norway) to a fixed level with 0 1 ml remaining in each well 2×10^3 ^{51}Cr-labeled target cells (see workshop) were added to the cultures, the force of the jet resuspending the cell pellet at the bottom of the well The plates were incubated for an additional 6 h and the supernatants collected using a Skatron multiple supernatant collection system (Skatron A/S, Lierbyen, Norway) The effector to target cell ratios were assessed from the number of effector cells added to each well on day 0 and the number of targets (2×10^3) added on day 6 (E/T usually 50/1 — 6 25/1), % cytotoxicity was calculated as in the workshop, using spontaneous release (targets + supplemented medium) and maximum release (targets + 5% Triton X) harvested as above after 6 h of incubation

3 — *Carboxyfluorescein labeling of target cells*

This technique was performed by Leiden 2 Details on target cell labeling have been published elsewhere (Bruning et al 1980)

Briefly, target cells were labeled by incubation in a 37°C waterbath for 15 min in a carboxyfluorescein-diacetate solution in RPMI (final concentration 40 μg/ml) After washing with RPMI-serum, 4000 target cells were added to the wells of Terasaki type microtest plates and fixed to the bottom by centrifugation Effectors were added and the fluorescence per well read in an automated microfluorometer After a 4 h incubation the released carboxyfluorescein was removed by washing and fluorescence of the remaining targets determined in the fluorometer From the difference of fluorescence between test and control wells the degree of cyto toxicity could be calculated

All other procedures were performed using the SE CML (Table 1)

Common Reagents used in Workshop III

1 *PBL* Unsensitized PBL from four selected blood donors (1, 2, 3, 4) were collected, frozen and shipped from Aarhus The donors had been fully HLA—A, B, C, D/DR and red cell antigen phenotyped and were selected in such a way that CTLs raised among them, in a checkerboard fashion, would yield cytolysis both explicable and inexplicable from our present knowledge of HLA (Kristensen et al 1974, 1976) Kristensen & Grunnet 1975) These cells were also those tested during CML-Workshop II (Bradley et al 1980)

2 *Primed PBL (1/2x, 1/3x, 1/4x, 4/1x 4/2x, 4/3x)* Cells primed *in vitro* in the Aarhus laboratory, according to the standard technique (Table 1), for 10 days were frozen and shipped to the other centers

3 *Human serum* Pooled AB serum was prepared from four male blood donors, screened as negative for lymphocytotoxic and red cell antibodies (A gift from Aarhus)

4 *PHA M* A common batch produced by Difco Co (0528—57) was used by all centers

5 *Radioisotope* ^{51}Cr as Na_2 $^{51}CrO_4$ (500 mCi/mg Cr, 5 mCi/ml) from the same production date was provided as a gift by Amersham (Amersham Co , England)

6 *Culture medium* RPMI 1640 (Gibco Co) supplemented with L-glutamine from the same production lot was provided as a gift from Grand Island Biological Company (in France, Flobio, S A)

Format of CML Workshop III

The format of the practical part of the Workshop is given in Table 2 PBL were collected in Aarhus and shipped on dry ice as either unprimed or primed (10 days) cells Primary cultures were established on day 0 followed by secondary cultures on day 1 Secondary cultures were restimulated either specifically (e g 1/2x ← 2x) or unspecifically by PWM Target cell cultures were established on day 3 and stimulated with PHA Cytotoxicity was measured on day 6 using the same target cell cultures for both 1° and 2° CTLs

For practical reasons, growth with conditioned media was initiated with the remaining CTL on day 6, hence requiring CML testing on different target cell cultures

Results

I *General Experiences*

The adoption of these techniques by the participating laboratories required changes for each group, although these were variable from laboratory to laboratory, and such changes led to individual experiences and difficulties in the III European CML Workshop

The following outlines the general assessment of the collaborating groups concerning their experiences with the standard protocol

A Recovery of cells after thawing of shipped standard reagents

1 *Unprimed cells* No standard technique was designated for the thawing of fr cells Despite variations in techni however, uniformly high recoveries cells with high viabilities were found b groups (Table 3) It was felt by the var groups that the method of cryopreserva used in Aarhus was excellent and that ment was not detrimental to t reagents

2 *Primed cells* In contrast to the suc with the unprimed cells, all groups, ex Aarhus, had relatively low recoveries viability in the shipment of primed c that might be overcome, in future te of 2° CML, by performing the I sensitizations in local centers rather th one laboratory providing cells An alt tive possibility to be considered variation in the local procedures determining recovery and viability cryopreserved cells

B Determination of recovery and viabi of cells

1 *Trypan blue or eosin dye exclusio* was felt by most groups that utilizatic such dyes which are excluded by v cells is a useful criterion for assessmer actual cell recovery, but that it is nc accurate assessment of functional reco since only a subpopulation of viable may be involved in the lytic process

2 *Phase contrast microscopy* An altern mean of distinguishing live from dead is their examination by phase con microscopy Again, this may be an acc means for assessment of viability cannot be used to ascertain the recove functional capacity

3 *Lytic unit assessment* of functi activity based on linear regression ana

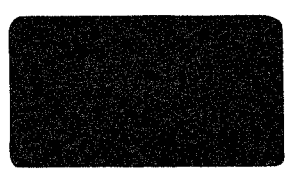


Table 2
FORMAT OF CML WORKSHOP III

		Day 0	Day 1	Day 3	Day 6	Day 17	Day 20		
PREPARATION OF CELLS FOR AARHUS SHIPMENT	All Labs	1°MLC- -	2°MLC- - -	TC/PHA- - -	CML*			Submission of data to Marseille	Third European CML Workshop meeting Marseille
	Aa		2°MLC- - - - - - - - - - -		CML†				
	Ma								
	Ma				CTL-CM- - -	TC/PHA- - -	CML§	Central analysis Marseille/Aarhus	

*Unsensitized PBL were thawed and primary (1°) MLC combinations were established on day 0. Primed cells, received from Aarhus, were thawed and restimulated in secondary (2°) MLC cultures on day 1. Target cell (TC) ampules were thawed on day 3 and stimulated with PHA. Cytotoxic from 1° and 2° CML were tested on day 6 in the ^{51}Cr-cell-mediated lympholysis (MCL) assay.

†The laboratories of Aarhus and Marseille thawed additional aliquots of primed cells which were restimulated with pokeweed mitogen (PWM) and tested in CML on day 6.

§The laboratories of Marseille and Munich initiated cytotoxic cells obtained from 1° and 2° MLC with conditioned medium (CM) containing T cell growth factor. These cells were expanded in culture for 14 days and tested in CML with target cells prepared 72 h in advance

Table 3a
Recovery of cells after thawing of shipped standard reagents (a) *Unprimed cells*

	Donor Number	Ar	Lei 1	Lei 2	Ma	Mu
% Viable	1					94
	2	N D	> 90%	> 90%	> 95%	93 5
	3					93
	4					93
Recovery†	1	7 3	7 0	6 8	9 1	6 1
	2	9 5	8 0	6 8	6 3	8 0
	3	8.2	8 2	8 0	8 3	8 0
	4	7 9	6 4	7 3	8 2	6 8

N D. Not done

†The concentration per frozen vial was $\simeq 10 \times 10^6$ cells in 1 ml Results are expressed as the av number $\times 10^{-6}$ of cells recovered per thawed vial

of CML activity prior to and following cryopreservation. Recovery of functional activity in cryopreserved samples can be defined by comparing the lytic unit for CTL samples prior to and following cryopreservation

C *Recovery of cells after MLC sensitization in the local laboratories*

1 *1° MLC sensitization* The starting concentration of responding and stimulating cells was stipulated in the standard protocol, but culture vessels to be utilized were left to individual judgment after determination of cell recovery from the cryopreserved reagents This led then to variation in the standard technique, with some laboratories culturing between 5 and 10×10^6 responding cells in upright standing culture flasks, with others culturing at 1×10^6 responding cells per well of a Cluster macrotiter culture plate (Costar), and still others using a combination of these two vessels. In terms of final recovery after the 6-day sensitization period, however, the results were remarkably uniform (Table 4a). Cell recovery from Oslo could not be determined because of their variation from the stan technique.

2. *2° MLC sensitization* In spite of the recoveries of primed cells from the c preserved ampules, all groups observed percentage yields after specific rest lation (Table 4b)

3. *Pokeweed mitogen restimulation primed cytotoxic cells* As an alternati restimulation of primed cytotoxic with specific γ-irradiated stimulating several laboratories received addit numbers of primed cells for restimul with PWM. Two variations in this prot were used by Aarhus and Marseille first used PWM at 1% final concentr with stimulation for 4 days (Mawas 1975). Marseille used 1% PWM with addition of autologous, γ-irradiated st lating cells (Charmot et al 1980) In cases high yields of cytotoxic cells functional activity were recovered.

4 *Expansion of CTL by growth in presence of Conditioned Medium* (Preliminary results were also obtained

Table 3b
(b) *Primed cells*

CML COMBINATION	Ar %V	Ar Rec	Ar %Rec	Lei 1 %V	Lei 1 Rec	Lei 1 %Rec	Lei 2 %V	Lei 2 Rec	Lei 2 %Rec	Ma %V	Ma Rec	Ma %Rec	Mu %V	Mu Rec	Mu %Rec	Os %V	Os Rec	Os %Rec
1/2x	[1]	4.9[2]	54[3]	Poor	1.3	14	Poor	2.9	32	80	3.2	36	75	2.1	21	22	2.5	28
1/3x		6.4	64		0.9	9		3.1	31	15	7	7	73	2.1	22	20	2.3	23
1/4x		5.2	52		1.7	17		2.4	24	50	2.4	24	85	1.9	20	20	2.1	21
4/1x		5.3	59		3.6	40		5.1	57	80	4.4	49	88	2.6	26	26	2.6	29
4/2x		4.6	58		2.7	34		4.7	59	80	2.9	36	82	1.8	18	34	2.6	33
4/3x		5.2	61		2.7	32		3.7	44	80	3.9	46	83	2.0	20	27	1.5	18

[1] Viability not assessed with vital dye exclusion.
[2] Results expressed as number of cells (10^{-6}).
[3] Calculated on the basis of the number of cultured small lymphocytes frozen in Aarhus ($8-10 \times 10^6$).

Table 4a

Recovery of cells after MLC sensitization in local laboratories (a) *Primary MLR*

TEST COMBINATION	%V	Ar Rec	%Rec	%V	Lei 1 Rec	%Rec	%V	Lei 2 Rec	%Rec	%V	Ma Rec	%Rec	%V	Mu Rec	%Rec	Os Os
1/2x	NT	18 0[1]	180[2]	good	25 4	254	good	19 2	148	> 90	20 0	167	92	19 8	264	NO DATA[3]
1/3x		20 3	203		16 9	169		13 9	126		19 8	165	95	17 1	228	
1/4x		21 1	211		13 6	136		20 3	185		37 6	313	90	20 7	276	
4/1x		15 9	159		13 6	136		13 7	125		19 0	158	77	13 8	184	
4/2x		14 8	148		12 6	126		10 1	101		15 2	127	73	13 7	144	
4/3x		15 6	156		9 3	93		11 5	105		12 4	103	74	13 3	140	
		(Flask)			(Flask)			(Flask and cluster)			(Cluster)					

[1] Results expressed as number of cells ($\times 10^6$)

[2] Based on the initial number of responder cells = 100%

[3] Due to a different technique

NT = not tested

Table 4b
Secondary MLR

TEST COMBINATION	%V	Ar Rec	%Rec	%V	Lei 1 Rec	%Rec	%V	Lei 2 Rec	%Rec	%V	Ma Rec	%Rec	%V	Mu Rec	%Rec	Os
1/2x	NT	21.6[1]	309[2]	Many	2.4	185		9.1	314		NT[3]		64	7.6	362	NO DATA[4]
1/3x		27.8	397	dead	2.0	222		10,3	332				75	9.5	452	
1/4x		23.1	330	cells	3.0	176		10.2	425				70	9.6	500	
4/1x		27.8	397		5.6	156	8.	8.2	161				29	2.6	100	
4/2x		26.1	373		11.6	430		10.5	223				69	9.1	500	
4/3x		33.8	483		14.5	537		9.3	251				72	5.2	260	
		(Flask)			(Cluster)			(Cluster)						(Flask)		

[1] Number of cells.
[2] Based on the input of responder cells = 100%.
[3] Marseille used PWM expansion only.
[4] Different technique.

Table 4c
PWM expansion of primed cells

TEST COMBINATION	NT	Ar Rec	%Rec	%V	Lei 1 Rec	%Rec	%V	Lei 2 Rec	%Rec	%V	Ma Rec	%Rec	%V	Mu† Rec	%Rec	%V	Os Rec	%Rec
1/2x	%V	2.5	83		NT			NT		> 90	13.0	406	80	4.3	204		NT	
1/3x		3.2	107								9.0	1286	60	2.9	138			
1/4x		3.0	100								13.0	542	32	2.6	137			
4/1x		3.8	127								19.4	441	50	1.3	50			
4/2x		3.4	113								12.6	434	58	5.3	294			
4/3x		3.4	113								23.4	600	21	1.4	70			
		Flask No autologous feeder									Flask autologous feeder (-irradiated 3500R)			Flask no autologous feeder				

Same legend as in (a) and (b).
†Cultures infected.

two laboratories testing the method of continued growth of CTL and their expansion by T cell growth factors present in conditioned medium. CTL obtained from both primary and secondary cultures MLC were tested after 14 days of culture in the presence of CM. CTL with very strong activity could be obtained although cytotoxic activity was not found in all combinations that were successfully expanded with TCGF (data not shown).

D. *Target cells*

1. *Ficoll flotation after* ^{51}Cr *labeling.* According to the standard protocol, labeled target cell preparations were to be layered over ficoll to separate live from dead and clumped cells. It was felt that this technique might reduce the level of spontaneous release of ^{51}Cr. The experience in this Workshop was that this procedure consumes time, and apparently did not enhance the results. The laboratories uniformly reported presence of very few clumped or dead cells in the starting populations. It was noted by some groups, however, that according to individual experience this is a valuable approach when the starting populations contain considerable numbers of dead cells. Some laboratories noted that no cells passed through the ficoll interface and suggested that this may have been due to the PHA agglutination of the target cells. It was suggested that this problem might be overcome by first washing the target cells in medium before layering over ficoll gradients. It was questioned whether DNase would be beneficial for the reduction of spontaneous release values and the elimination of clumped cells In the experience of Aarhus this was not observed.

2. *Triton X determination of maximum release of* ^{51}Cr. According to the standard protocol, maximum release was determined by detergent lysis of target cells. Several laboratories had difficulty with this procedure, failing to obtain solutions of lysed target cells, instead they obtained colloidal suspensions. In discussion it was ascertained that this was due to the use of 100% Triton X instead of a 5% solution of Triton X in H_2O. In most laboratories, very little variation between the values of maximum release as determined by Triton X detergent lysis and total cpm of ^{51}Cr was found on a per cell basis. Thus, it appears that MR values must not be determined and calculations can be based on total cpm. These results indicate that there is not an increament of ^{51}Cr counts that is non-releasable from target cells, as has been suggested previously. This observation, however, may be dependent upon the method used for the determination of MR.

E. *Harvesting of supernatants from microtiter wells.*

The utilization of V-bottomed plates in the standard protocol led to some difficulties by various groups regarding means for harvesting standard aliquots of supernatant. Some local methods utilized commercial filter systems for round-bottomed plates or testing in larger volumes allowing supernatants to be decanted after centrifugation. With the V-bottomed plates there was a dilemma regarding a suitable means of supernatant collection. Some groups noted harvesting by hand to be tedious and time consuming and potentially increasing spontaneous release. Leiden 1 successfully used the commercial filter systems in the V-bottomed plates while Marseille used a locally designed multiple harvester which has 961cc syringes set into a plexiglass

frame which could simultaneously harvest the samples of 0 1 ml/well of an entire plate

F *Alternative CML techniques*
In addition to the standard protocol used by four participating groups, two additional approaches were tested with the same reagents and could be compared in Workshop III

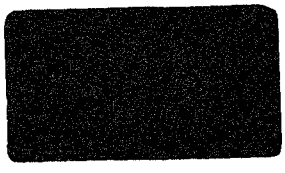
1 *Generation of CTL in microtiter plates* As presented later in the section on data analysis, this approach led to variance in the results compared to those of the standard protocol The technique did, however, reveal that quantitative evaluation of CML activity could be made with few numbers of starting cells It was felt that development of this approach may be beneficial in the clinical situation when tests of young children, dialysed, transplanted, aplastic, or immunodeficient patients must be performed

2 *Assessment of cytotoxic activity using carboxyfluorescein as a viability label* The second technique tested in parallel with the standard protocol was that of utilizing the viability dye, carboxyfluorescein, instead of ^{51}Cr for the determination of destruction of target cells Basically, in this technique (Bruning et al 1980), target cells are labeled by incubation with the diacetate derivative which is taken up only by viable cells, after which the dye is liberated in the cell by intracellular hydrolysis The target cells are fixed by centrifugation to the well bottom of Terasaki type microtest plates Effector cells are added to the targets and total well fluorescence is determined before the incubation period using an inverted epi-illuminated fluorescence microscope with photomultiplier and a stage driven by stepping motors assembly is controlled by a mini-com After incubation with CTLs for period the released dye is removed a residual fluorescence is measured i automated fluorometer Loss of escence is then used as a measure c lysis of the target cells Comparable sp city was found using this new tech (cf below) It was felt that great pot lies in the utilization of this approa the future if the equipment bec commercially available It eliminate requirement for radioactive material automates the reading of results, red normal harvesting and counting times 1 to approximately 30 sec, for 60 san Comparison of quantitation and speci with the Workshop standard protoc presented later

II *Data Analysis*
Data were submitted from each indiv laboratory in three forms (1) raw obtained with each CTL tested against different target cells at each of six dilu (Oslo, submitting on fewer dilutions) data calculated and expressed as re percentages according to formulas of individual laboratories listed in Tabl and (3) qualitative assignment of po and negative responses according to judgment

Joint analysis was performed in Mar and Aarhus and is outlined below

A *Preliminary calculations*
1 All submitted data were graphi plotted to assess the (curvi)-linear relat ship between the independent vari (E/T ratio) and the dependent one (rel percentages) For most positive co nations an S-shaped curvi-linear relation was observed A priori it is expected



logarithmic transformation of the X-values (i e E/T ratios) should yield a linear relationship Consequently, to simplify subsequent regression analysis E/T ratios were transformed to $\log_{10}$ In most instances this changed a curvilinear relation ship into a linear one, at the same time increasing the proportion of variance of the dependent variable explained by the X-value (i e $\log_{10}$ E/T), and making the distribution of the deviations from the regression line fit the normal Assessment of data using square-root and reciprocal transformations both on X- and/or Y-axis values was unsuccessful

2 Based on data transformed according to (1), LU_{30} and release percentages at E/T ratio = 30 were extra- or intrapolated

3 For the analysis of variance, release percentages were Log_{10} transformed

B *Main analysis*

1. *A*nalysis *o*f *V*ariance (Anova) was made based on (a) $\log_{10}$ release percentages at E/T ratio 30/1, (b) LU_{30} (*L*ytic *u*nits i e the number of effectors needed to obtain 30% target cell lysis) Anova was performed to identify the variance components and their interactions both within and among laboratories

2 Linear regression analysis of release percentages with varying $\log_{10}$ E/T ratios were used to define the regression equations obtained by different laboratories when testing supposedly identical CTLs on identical targets

Using least square methods, straight lines were approximated and defined by the regression equation

$$\text{Release \%} = a + b \log_{10} \text{E/T}$$

(where b = the regression coefficient = the derivative of the function, a = the intercept of the regression line with the Y axis = the release % at E/T = 0)

The statistical significance of b was ascertained using a t-test under the null hypothesis that b = 0

3 *A*nalysis of *Cova*riance (Ancova) was used to compare the regression equations obtained within and among different laboratories using supposedly identical CTLs on identical targets

4 2 × 2 comparison analyses were used to compare qualitative assignments made locally and jointly

For basic references to the statistics used, cf Sokal & Rohlf (1969) and Bradley et al (1980)

For obvious reasons it is impossible to present all data and calculations performed in the joint analysis The data given are thus summaries *A total set of data may be obtained from the group*

Table 5
Comparison of target cell data W III

	1*	2*	3*	4*	5*
Aarhus					
SR	1434	1945	1021	2690	971
MR	5813	6385	4640	8137	4466
Leiden 1					
SR	1356	1132	769	1146	1065
MR	5534	6198	4516	4657	7169
Marseille					
SR	483	600	440	1118	613
MR	6281	3944	4080	8393	6453
Munich					
SR	582	323	173	789	235
MR	3015	3580	2775	4176	2474
Oslo					
SR	526	580	572	810	564
MR	2359	2936	3662	4139	3371

SR = Spontaneous release cpm (mean of triplicates)
MR = Maximum release cpm (mean of triplicates)

Results of Joint Analysis

1 *Raw data – cpm*

One of the major findings from Workshop II was that there were marked differences in the ways that different laboratories handled identical target cells, as opposed to their handling of effector cells Reasons for these differences might be the use of differential ^{51}Cr products, including different batches, labeling amounts and labeling procedures In Workshop III these parameters were standardized and the results in terms of spontaneous release and maximum release cpm are given in Table 5

When comparing the cpm among laboratories and targets considerable variance is observed However, when the cpm concerning the duplicate targets (3 and 5) are compared within a given laboratory a high degree of reproducibility is found Consequently, these findings may, at least partly, be attributed to differences between laboratories in the assessment of spontaneous and maximum releases, as well as harvesting and counting procedures (cf Table 1)

2 *Analysis of variance (Anova)*

Both release percentages at E/T = 30 and LU_{30} were subjected to Anova in which the following questions were asked (cf Table 6)

(a) How big is the variance component at the different levels?

(b) Was there a significant difference in the way in which different laboratories handled their effector cells?

(c) Was there a significant difference in the way in which different laboratories handled their target cells?

(d) Was there a significant difference in the way in which effector cells interact with the targets? This of course was expected since it reflected the biol the CML

(e) Were any laboratories addin tively more to the total variance a pared to others? This was evaluat reperforming the analysis following s ive exclusion of individual laboratori

From Table 6 (where the data $\log_{10}$ release percentages are present can be seen that the *major signi* variance found with the Wor technique is that from the CTL and interaction, i e the biological varian CML In addition there is variance i way different laboratories treat targe CTLs The latter (laboratory versus can be attributed mainly to one labor (Oslo), which used a different technique (cf Table 1 and above) variance by the laboratory and target action could be attributed to Leid which used the CF-technique, and Mars However, the "target cell prob disclosed itself in all centers, in as mu the variance components including target component were always the larg

Due to the overall significance of interaction variance no main effect vari could be tested for statistical signific Anova-calculations performed with I yielded fully comparable variance c ponents and significance values furnished no new information (data shown) Comparing the overall vari components of Workshop III to t components obtained using local techni during Workshop II (Bradley et al 198 is, however, quite evident that much n variance has accumulated in the CTL target interaction group, pointing t more uniform and stable technique wh more clearly discloses the biolog interaction

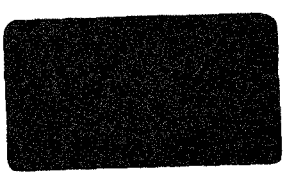


Table 6
CML-Workshop III combined analysis – three-way analysis of variance. Log. 10-transformed release percentages

Source of variation	Variance components (significance) ALL	-Ar	-Lei 1	-Lei 2	-Ma	-Mu	-Os
Laboratories	3.2% (–)	1.6% (–)	2.7% (–)	3.5% (–)	3.1% (–)	2.0% (–)	1.0% (ns)
CTLs	2.4% (–)	3.1% (–)	2.5% (–)	3.6% (–)	2.7% (–)	3.8% (–)	2.8% (–)
Targets	2.8% (–)	2.7% (–)	4.2% (–)	3.6% (–)	3.3% (–)	2.2% (–)	2.9% (–)
LABS & CTLs	4.7% (••)	6.2% (••)	5.6% (••)	3.1% (••)	2.4% (•)	4.7% (••)	1.5% (ns)
LABS & Targets	8.0% (••)	7.1% (••)	7.2% (••)	6.4% (•)	6.1% (•)	9.6% (••)	3.3% (•)
CTLs & Targets	70.0% (•••)	69.4% (•••)	70.4% (•••)	68.9% (•••)	72.3% (•••)	69.1% (•••)	76.6% (•••)
LABS & CTLs & Targets	8.5% (–)	9.9% (–)	7.4% (–)	10.9% (–)	11.3% (–)	8.6% (–)	11.9% (–)

• : $P \leqslant 5\%$
•• : $P \leqslant 1\%$
••• : $P \leqslant .1\%$
ns : not significant
– : cannot be tested.

3. *Analysis of covariance (Ancova)*
This analysis was applied in order to compare the regression equations obtained both within and among laboratories when identical CTLs were tested against identical targets in etither 1° or 2° CML, i.e. whether the biology of the different tests was identical (The data from Oslo were excluded from this analysis).

The following analyses were made:

(1) within laboratories on duplicate targets (3 and 5);

(2) within laboratories in 1° and 2° CML;

(3) among laboratories with regard to specific CTL-target combinations in either 1° or 2° CML.

The results were uniformly disappointing, showing differences significant at the 1% level.

Thus, even with standardized reagents using a standardized technique, a single laboratory is not able to fully describe the biological interaction and hence the immunology of CML.

It is easy to speculate that during freezing and thawing, MLC incubation or target cell proliferation, not all active clones survive and proliferate equally, but rather that some clones are already lost from the lymphocyte population prior to culture in a stochastic fashion varying from laboratory to laboratory. Since, however, major specificities are maintained and almost uniform positive and negative assignments can be obtained (cf. below) important (high affinity ?) clones must be selected actively during CTL sensitization whereas peripheral (low affinity ?) clones undergo stochastic selection. During target-cell culture with PHA no active selection should occur leading to more variability (the target cell

problem) These findings and speculations underline some of the difficulties which must be considered when CTLs are to be used as immunogenetic typing reagents

4 *Joint analysis based on linear regression analysis and positive slope assignment*

(a) *Basis for linear regression* Plotting percent release versus dilutions of CTL gives an S-shaped curve When the dilution of CTL (E/T) is transformed to $\log_{10}$ value a straight line should be generated Using least square methods, straight lines can be approximated for these values of X and release percentages for the values of Y If these have a significantly positive slope, tested as described above, the information can be used to quantify the CML response If too few dilutions are compared the possibility to assign a significant b value is decreased (cf above)

(b) Slope of the regression line may be used to assess the presence and subsequently the strength of a cytotoxic response For positive CTLs it is expected that the slopes will be positive, however, some laboratories observed no (or negative) slopes, the basis of which is discussed below

(c) Combined assignment based solely on a significantly positive slope was then used to judge the data of all laboratories The variations between the first local assignments and these new assignments were discussed and each laboratory detailed its own exceptions and the basis for original local assignment

5 *On the basis of these discussions the problems below were encountered*

(a) In some cases significance by positive slope may not be obtained if too few dilutions of CTL are tested In the standard protocol for this experiment the use of six dilutions in duplicate was suggested

(b) A positive slope may also fail reach significance when cytotoxic activi reaches a plateau with most of the dilutio or, in the case of Leiden 2, when cytotox activity decreases with the highest E ratios Similarly there may be a plateau the lowest level if the first dilutions do n exceed the detection level This cou then cause false elimination of positi reactions

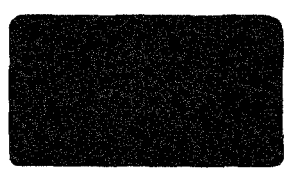
(c) Negative slopes were observed some combinations of CTL against th autologous target cells The basis these observations was discussed First, t might be due to a stabilizing effect increasing numbers of CTL on the spo taneous release of ^{51}Cr from the tar cells This then leads to the dilemma the most suitable control for spontaneo release (i e AB_x on A, unsensitized A A, AA_x on A or, as was commonly us here, A in medium alone) The latter l been conventionally adopted by m laboratories to save on reagents

(d) Alternatively, positive slopes may a be observed when the CTL is test with its autologous target cell St activities should not automatically ignored or ascribed merely to techni artefacts They may reflect recognition antigens of lymphocyte subpopulations perhaps of anti-idiotype responses

6 *Conclusion of the Joint Data Analysis Using Regression and Slope Data*

(a) Positive assignments cannot be m solely on the basis of significantly posit slopes Not all reactions are transformed linearity by the logarithmic approa Some difficulties may be solved by usin minimum number of E/T dilutions (grea or equal to five)

(b) Since these approaches may not work



is necessary to accept a release percentage value greater than or equal to 10% at any dilution as indicating a positive response This perhaps can be more safely assumed if duplicate CTL cultures are also tested

(c) For combinations tested at very low dilutions showing a slope not significantly positive extrapolations should be made to E/T ratios = 50 A release percentage greater than or equal to 10% should then be considered positive

(d) The observation of a positive slope cannot always be taken as a means of determination since immunologically negative controls may show significantly positive slopes (cf 5 d)

Workshop Criteria for Assignments and Exclusions

As a consequence of the above mentioned experience and discussion the following criteria were accepted for the joint assignments and exclusions

A *Positive assignments were based on*

1 Significantly positive slope of regression lines of release percentages on $\log_{10}$ E/T, and/or

2 Release percentage > 10% in any dilution tested If a CTL was tested only at E/T dilutions below 50/1 extrapolation was performed An extrapolated release percentage > 10% at E/T = 50/1 was then considered positive

B *Exclusions were based on*

1 A positive assignment (cf above) on autologous targets (e g 1/2x → 1)

2 A negative assignment (cf above) on the specific target (e g 1/2x → 2)

Table 7
CML Workshop III — combined assignments

	TARGET 1						TARGET 2						TARGET 3						TARGET 4						TARGET 5					
CTLs	A	L1	L2	Ma	M	O	A	L1	L2	Ma	M	O	A	L1	L2	Ma	M	O	A	L1	L2	Ma	M	O	A	L1	L2	Ma	M	O
1° 1/2x	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	−	+	+	+	−	+	−
2° 1/2x	−	−	−	−	−	/	+	+	+	+	+	/	+	+	+	+	+	/	+	+	+	+	+	/	+	+	+	+	+	/
1° 1/3x	−	−	−	−	−	/	+	+	+	+	+	/	+	+	+	+	+	/	+	+	+	+	+	/	+	+	+	+	+	/
2° 1/3x	−	/	−	−	−	/	+	/	+	+	+	/	+	/	+	+	+	/	+	/	+	+	+	/	+	/	+	+	+	/
1° 1/4x	−	/	−	−	−	/	+	/	+	+	+	/	+	/	+	+	+	/	+	/	+	+	+	/	+	/	+	+	+	/
2° 1/4x	−	/	−	−	−	−	+	/	+	+	+	+	+	/	+	+	+	+	+	/	+	+	+	+	+	/	+	+	+	−
1° 4/1x	+	+	+	/	+	+	−	+	+	/	+	−	−	−	−	/	−	−	−	−	−	/	−	−	−	−	−	/	−	−
2° 4/1x	+	+	+	+	/	+	−	+	−	+	/	−	−	−	−	−	/	−	−	−	−	−	/	−	−	−	−	−	/	−
1° 4/2x	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2° 4/2x	/	+	+	+	+	−	/	+	+	+	+	+	/	−	−	−	−	−	/	−	−	−	−	−	/	−	−	−	−	−
1° 4/3x	−	+	−	+	+	+	−	−	+	+	+	−	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	−
2° 4/3x	−	−	/	−	−	/	−	−	/	−	−	/	+	+	/	+	+	/	−	−	/	−	−	/	+	+	/	+	+	/

\+ = positive assignment
− = negative assignment
/ = excluded

Table 8a
CML-Workshop III – 1° CML 2 × 2 analysis of combined assignments

	++	+−	−+	−−	N
Ar vs Lei 1	13	0	2	10	25
Lei 2	17	0	2	11	30
Ma	13	3	2	7	25
Mu	16	0	3	11	30
Os	5	4	1	10	20
Lei 1 vs Lei 2	14	1	1	9	25
Ma	10	3	1	6	20
Mu	15	0	1	9	25
Os	6	5	0	9	20
Lei 2 vs Ma	14	3	1	7	20
Mu	19	0	1	10	30
Os	5	6	1	8	20
Ma vs Mu	15	0	3	7	25
Os	4	3	1	7	15
Mu vs os	6	6	0	8	20
Excluded CTLs : Ar	: 1				
Lei 1	: 3				
Lei 2	: 1				
Ma	: 1				
Mu	: 1				
Os	: 3				

3. Direct indication from the laboratory in question (infection, not enough cells, technical failure, . . .).

2 × 2 Table Analysis of Combined Assignments

Based on the assignment criteria outlined above, all local assignments were reassessed leading to exclusion of some CTLs due to positive and/or negative control values, while others (locally excluded) were accepted into the analysis. The combined assignments are shown in Table 7.

Based on these assignments, 2 × 2 tables were constructed as shown in Table 8.

The conclusions are as follows:

1. 1° CML : good correlations, with Munich showing the broadest assignment of positive values (Table 8).
2. 2° CML : very few discrepancies excep with Oslo showing few positive reactio (Table 8b).
3. 1° versus 2° CML (Table 8c) : excelle agreement. There may be a tendency obtain more positives in the 1° CM than 2°, except for Marseille.

   Marseille as standard uses a 2° CM so may be better at this technique.
4. CFA versus ^{51}Cr : no major discre ancies with all other groups except Os in both 1° and 2° CML (Table 8d).
5. 2 × 2 analysis of reactions of all CT against duplicate targets : excelle agreement (Table 8e).

Table 8b
CML-Workshop III – 2° CML 2 × 2 analysis combined assignments

	++	+−	−+	−−	
Ar vs Lei 1	7	0	1	7	1
Lei 2	13	0	0	12	2
Ma•	15	0	1	9	2
Mu	14	0	0	6	2
Os	4	1	0	5	1
Lei 1 vs Lei 2	7	1	0	7	1
Ma•	10	0	0	10	2
Mu	8	0	0	7	
Os	2	2	0	6	
Lei 2 vs Ma•	15	0	1	9	
Mu	14	0	0	6	
Os	5	2	0	8	
Ma• vs Mu	16	0	0	9	
Os	5	3	0	7	
Mu vs Os	4	2	0	4	
Excluded CTLs : Ar	: 1				
Lei 1	: 3				
Lei 2	: 1				
Ma	: 1				
Mu	: 1				
Os	: 3				

•The result from Ma is not directly comparab as 2° CML was performed only using expansi of 1° CTLs with PWM and irradiated, autologo feeder cells. No specific restimulation v performed.

[redacted]

Table 8c
CML-Workshop III – 1° vs 2° CML 2 × 2 analysis of combined assignments

	++	+−	−+	−−	N
Ar vs Ar	15	0	0	10	25
Lei 1 vs Lei 1	10	1	0	9	20
Lei 2 vs Lei 2	15	1	0	9	25
Ma vs Ma	13	2	3	7	25
Mu vs Mu	16	2	0	7	25
Os vs Os	2	1	0	7	10
TOTAL	71	7	3	49	130

6. Specific 2° restimulation versus PWM : (fair agreement). Data not shown since available only from Aarhus and Marseille.

On the Specifications Detected by Workshop III CTLs

The cells circulated in this Workshop were identical to those circulated during Workshop II. An account of their total red and white cell phenotypes as defined by serology is given in the report from Workshop II (Bradley et al. 1980).

In terms of immunogenetic specificity the data of Workshop II were translated as follows:

1. Positive lysis was strongly correlated to sharing of HLA-A, B, C, DR serologically defined determinants between stimulator and target cells; however,

2. No lysis was observed in the presence of HLA-antigenic sharing between stimulator and target (five combinations).

3. Two combinations (4/1x → 2 and 4/2x → 1) gave positive CML in the absence of any known serologically defined antigen. This is in concordance with earlier findings (e.g. Kristensen et al. 1974).

In this Workshop III only the combinations from Workshop II 1/2x, 1/3x, 1/4x, 4/1x, 4/2x and 4/3x were repeated with the standard technique. The conclusions (1) and (3) above are clearly confirmed, while (2) is invalidated in as much as the one previously negative combination (1/3x → 2) retested is uniformly positive with the Workshop III standard technique (cf. Table 7). The

Table 8d
CML-Workshop III – CFA vs ^{51}Cr targets 2 × 2 analysis of combined assignments

	++	+−	−+	−−	N
1° CML					
Lei 2 vs Ar	17	2	0	11	30
Lei 1	14	1	1	9	25
Ma	14	3	1	7	25
Mu	19	0	1	10	30
Os	5	6	1	8	20
2°CML					
Lei 2 vs Ar	7	1	0	7	15
Lei 1	7	0	1	7	15
Ma	15	0	1	9	25
Mu	14	0	0	6	20
Os	5	2	0	8	15
TOTAL	117	15	6	82	220

Table 8e
CML-Workshop III – duplicate targets (3 vs 5) 2 × 2 analysis of combined assignments

	++	+−	−+	−−	N
1° CML					
Ar	4	0	0	2	6
Lei 1	3	0	0	2	5
Lei 2	4	0	0	2	6
Ma	3	1	0	1	5
Mu	3				
Os	0	1	0	3	4
2° CML					
Ar	4	0	0	1	5
Lei 1	2	0	0	2	4
Lei 2	3	0	0	2	5
Mu	4	0	0	1	5
Os	0	1	0	2	3
TOTAL	36	3	0	22	60

Table 9
CTL detecting new specificities activity in CML Workshop III

	1° CML of 4/1x and 4/2x with 1* and 2*			
4/1x				
Laboratory	b(1*)	Slope (1*)	b(2*)	Slope(2*
Aarhus	0.96°°	9.15	0.68	3.30
Leiden-1	0.96°°	17.02	0.96°°	9.40
Leidern-2	0.93°°	15.11	0.68	9.10
Munich	0.89°°	10.57	0.98°°	5.37
Oslo	0.98°°	19.51	0.93°°	6.78
4/2x				
Laboratory	b(1*)	Slope (2*)	b(2*)	Slope (1
Aarhus	0.90°	5.68	0.97°°	5.81
Leiden-1	0.96°°	11.67	0.97°°	12.1(
Leiden-2	0.96°°	16.12	0.82°	8.44
Marseille	0.98°°	10.08	0.38	1.5(
Munich	0.99°°	19.92	0.97°°	13.3<
Oslo	0.91°°	9.73	0.92°°	5.5;

° $P \leqslant 5\%$.
°° $P \leqslant 1\%$.

Workshop III data on CTLs 4/1x and 4/2x possibly detecting new specificities are given in Table 9.

The conclusion, therefore, that CTLs primed *in vitro* recognize HLA-coded determinants, but that these may well be different from the serologically defined determinants is still very pertinent.

It must, however, not be forgotten that standardizing our individual techniques on the ability to produce identical assignments may counterselect for virtues of local techniques which would enable identification of marginal or weak effects such as e.g. "the target cell problems".

General Conclusions and Future Goals

1. There was excellent recovery and viability in all groups regarding the unprimed responding and stimulating cells and the CTL recovered after *in vitro* stimulation.

2. Improvements are needed at the level of 2° responses, including freezing of primed cells, or alternatively doing this the local level.

3. All groups should aim to initiate stud of alternative means of stimulating CTL 2° reactions. This would include the u of PWM and special advice can be obtair from Marseille.

4. Hopefully, all groups will attempt make T-cell growth factor and try to gr CTL in such media. Samples for cc parison will be shipped from Munich , Marseille to all other groups and th laboratories will help with special advice

5. Techniques for cloning of human all active T-cells are now available (e.g. Mali et al. 1980). Local techniques experience will be circulated and compa locally.

6. New information can be obtained f analyzing various target cell populati Aarhus has developed a uniform techn for making B cell blasts (Johnsen 19

and it should be initiated in all laboratories The use of cultured T cells and other target cell types should be tested according to the interests of the individuals

7 Competitive inhibition using cold targets can be a means to overcome some of the difficulties that most groups encounter when using a panel of ^{51}Cr target cells Before the next Workshop, individual laboratories should experiment with this approach, e g using early and late PHA-blasts, EBV-cell-lines, T cell lines or platelets as cold competitors

8 In the standard technique it still appears that there is a "target cell problem" It was suggested that laboratories might try Tris or Hepes buffered medium for ^{51}Cr incubation and target cell washing It may be advisable to first distribute the CTL to the plates and return them to an incubator to readjust temperature and pH before adding the target cells

9 It is by now desired to expand the participation in the CML Workshops as long as the new participants are actively involved in the area of CML research and are willing to share their expertise on the same basis as the others

10 The immediate goal for CML-Workshop IV will be discussed within the group following local assessment of the questions raised above A preliminary decision has been made to place the next Workshop meeting in Leiden during September 1980

Acknowledgments

The group wants to thank Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, France, for providing excellent facilities during the workshop meeting and to express its gratitude to Colette Kourilsky, Marseille, for her expert secretarial assistance

Dr Mawas acknowledges the support of FLOBIO, Sa to the "Association pour la Recherche en Immunologie de Marseille-Luminy" for part of the Workshop social expenses

References

Albrechtsen, D , Arnesen, E & Thorsby, E (1979) Cell mediated lymphocytotoxicity directed against HLA-D gene products *Transplantation* **27**, 338–341

Bonnard, G D , Schendel, D J , West, W H , Alvarez, J M Maca, R D , Yasaka, K , Fine, R L , Herberman, R B , De Landazuri, M O & Morgan, D A (1978) Continued growth of normal human T lymphocytes in culture with retention of important functions In *Human Lymphocyte Differentiation Its Application to Cancer*, eds Serrou, B & Rossenfeld, C , pp 319–326 Elsevier/North Holland Biomedical Press

Bradley, B A , Charmot, D , Goulmy, E , Johnsen, H E , Kristensen, T , Mawas, C , Pfeffer, P , Schendel, D & Wank, R (1980) Histocompatibility typing by cell mediated lympholysis (CML) Workshop II Technical standardization *Tissue Antigens* **16**, 73–90

Bradley, B A , Edwards, J M , Dunn, D C & Calne, R Y (1972) Quantitation of mixed lymphocyte reaction by gene dosage phenomenon *Nature* **240**, 54–56

Bradley, B A , Goulmy, E , Schreuder, I & Van Rood, J J (1978) Targets for killer T cells In *Human Lymphocyte Differentiation Its Application to Cancer* eds Serrou, B & Rosenfeld, C , pp 231–240 Elsevier/North-Holland Biomedical Press

Bruning, J W , Kardol, M J & Arentzen, R (1980) Carboxyfluorescein fluorochromasia assays I Non radioactively labeled cell mediated lympholysis *J Immunol Methods* **33**, 33–44

Charmot, D , Malissen, B , Chiotto, M & Mawas, C (1980) Expansion of human lymphocyte population expressing specific immune reactivities II A comparison of immune reactivities in human T lymphocyte lines derived from allogenetically primed cultures

and maintained with lectins or conditioned medium *Tissue Antigens* **15**, 297–312

Eijsvoogel, V P , Bois, M J C J du, Meinesz, A , Bierhorst-Eijlander, A , Zeylemaker, W P & Schellekens, P T A (1973) The specificity and the activation mechanism of Cell Mediated Lympholysis (CML) in Man *Transplant Proc* **5**, 1675–1679

Feighery, C F & Statsny, P (1979) Specific inhibition of T-cell mediated cytolysis by alloantiserum against HLA–D-related (DR) antigens *Transplant Proc* **11**, 708–711

Goulmy, E , Blockland, E , Van Rood, J J , Charmot, D , Malissen, B & Mawas, C (1980) Production, expansion and clonal analysis of T cells with specific HLA restricted male lysis *J exp Med* **152**, 1825–1905

Goulmy, E , Termijtelen, A , Bradley, B A & Van Rood, J J (1976) HLA restriction of non HLA–A, –B, –C and –D cell mediated lympholysis (CML) *Tissue Antigens* **8**, 317–326

Goulmy, E , Termijtelen, A , Bradley, B A & Van Rood, J J (1977) H Y antigen killing by women is restricted by HLA *Nature* **266**, 544–546

Grunnet, N , Kristensen, T & Kissmeyer-Nielsen, F (1975) Cell mediated lympholysis in Man Patterns of killing power in relation to the HL A system *Tissue Antigens* **6**, 205–220

Grunnet, N , Kristensen, T & Kissmeyer Nielsen, F (1976) Cell mediated lympholysis in Man The impact of *HLA–C antigens Tissue Antigens* **7**, 301–309

Hirschberg, H , Skare, H & Thorsby, E (1977) Cell mediated lympholysis CML, a microplate technique requiring few target cells and employing a new method of supernatant collection *J Immunol Methods* **16**, 131–141

Histocompatibility Testing (1975) ed Kissmeyer Nielsen, F Munksgaard, Copenhagen

Histocompatibility Testing (1977) eds Bodmer, W F , Batchelor, J R , Bodmer, J G , Festenstein, H H & Morris, P J Munksgaard, Copenhagen

Johnsen, H E *(1980) Human B*-blast specific target determinants in CML a panel study *Tissue Antigens* **15**, 199–209

Jørgensen, F & Lamm, L U (1974) MLC – a micromodification of the mixed leukocyte culture technique *Tissue Antigens* **4**, 482–494

Kristensen, T (1978) Studies on the specifi of CML Report from a CML Worksh *Tissue Antigens* **11**, 330–349

Kristensen, T & Grunnet, N (1975) Cell Medi Lympholysis (CML) in Man Evidence a separate locus within the Major Histoc patibility Complex (MHC) and an Appr to CML typing *Histocompatibility Test* pp 835–844 Munksgaard, Copenhagen

Kristensen, T Grunnet, N , Jørgensen, F , La L U & Kissmeyer Nielsen, F (1976) Mediated Lympholysis in Man An attemp type with cytotoxic lymphocytes *Ti Antigens* **8**, 299–316

Kristensen, T , Grunnet, N & Kissmeyer Nier F (1976) Cell Mediated Lympholysis in An attempt to type with cytotoxic lym cytes *Tissue Antigens* **8**, 299–316

Kristensen, T , Grunnet, N & Kissmeyer Nie F (1974) Cell mediated lympholysis in Occurrence of unexpected HL A (LA Four) irrelevant lympholysis *Tissue Ant* **4**, 378–382

Malissen, B , Charmot, D , Goulmy, E , Block E , Liabeuf, A , Goridis, C , Mawas, Kourilsky, F M (1980) Alloreactive, n functional human T cell clones expr cytotoxic or primed lymphocyte typi well as restricted immunogenetic specifi *New trends in Immunology and C Immunotherapy*, eds Serrou, B & Rose C Elsevier/North Holland, Amsterda press

Malissen, B , Charmot, D , Liabeuf, A & M C (1979) Expansion of human lymph populations expressing specific imr reactivities I Differential effects of v lectins on the expression of allor cytotoxicity in primed cells *J Immuno* 1781–1787

Mawas, C , Christen, Y , Legrand, L & D J (1973) Cell Mediated Cytotoxicity i human lymphocytes comparison betw vivo immunization and in vitro sensiti *Transplant Proc* **5**, 1691–1695

Mawas, C , Christen, Y , Legrand, L , Sas M & Dausset, J (1974) Cellular and h response against determinants other th classical HL A specificities *Transpla* **18**, 256–266

Mawas, C , Sasportes, M , Charmot, D , Cl Y & Dausset, J (1975) Cell mediated ly lysis in vitro Independence of

lymphocyte reactions and T-cell mitogen responses from the in vitro generation of cytotoxic effectors in primary immunodeficiency diseases. *Clin exp. Immunol* **20**, 83.

Mempel, W., Grosse-Wilde, H., Baumann, P., Netzel, B., Steinbauer-Rosenthal, I., Scholz, S., Bertrams, J. & Albert, E. D. (1973) Population genetics of the MLC response : typing for MLC determinants using homozygous and heterozygous reference cells. *Transplant Proc.* **5**, 1529–1534.

Schendel, D. J., Bonnard, G. D., Wank, R. & Dupont, B. (1978b) Proliferation and destructive capacities of human cultured T cells. *Transplant Proc.* **10**, 933–935.

Schendel, D. J., Wank, R. & Bonnard, G. D. (1980) Genetic specificity of primary and secondary proliferative and cytotoxic responses of human lymphocytes grown in continued culture. *Scand. J. Immunol.* **11**, 99–108.

Schendel, D. J., Wank, R. & Dupont, B. (1978a) Examination of HLA genetic fine structure and complementation using cytotoxic lymphocytes. *Eur. J. Immunol.* **8**, 634–640.

Schendel, D. J., Wank, R., Hansen, J. A. & Dupont, B. (1977) Cell-mediated lympholysis in man : identification of a common target determinant associatied with HLA–B12 and Bw35. *Transplant. Proc* **9**, 1777–1780.

Sokal, R. R. & Rohlf, F. J. (1969) *Biometry*. W. H. Freeman and Company, San Francisco.

Address:
Dr. Tom Kristensen
Centre d'Immunologie
INSERM – CNRS
70, Route Léon Lachamp
13288 Marseille case 90b
Cedex 9
France