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Histocompatibility Typing by Cell Mediated Lympholysis (CML): Workshop II† Technical Standardization

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The concept of cellular typing by cell mediated lympholysis (CML), in which cytotoxic lymphocytes are used to identify cell surface structures on target cells, has emerged over the last decade as it has become apparent that foreign structures on target cells can be recognized by T cells (Cerottini et al. 1979a, b, Sondel et al. 1975). It is not yet clear, nor should it be assumed, that target molecules recognized by CML are also recognizable by classical antibody. Such a concordance or discordance will only become apparent when more is known of the immunological topography of cell surface structures. Tentative specificities, however, have been identified by CML in man which have, as yet, not been recognized by classical antibody (Mawas et al. 1973, 1976, Kristensen et al. 1974, Goulmy et al. 1976, 1977, Bradley et al. 1978). Moreover, one can generate CML effector cells as reagents *in vitro* from native or immunized PBL and more recently it has become possible to propogate them in long term culture and ultimately derive clones (Bonnard et al. 1978, Charmot et al. 1979, Schendel et al. 1979a, Schendel & Bonnard 1979, Malissen et al. 1979). These

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Table 1
Serologically defined foreign phenotypes seen by the primed cells

Resp /Primary stimulator	HLA	Non-HLA
1/2	A2, 3, B5, 7, DRw2	M, S, k
1/3	A28, 3, B7, w35, Cw4, DRw7	c E, M, S, k
1/4	A28, 3, B5, 7, DRw2	M, k
2/1	Aw19, B8, 14, DRw6	A, N, K, Fy^a, Jk^a
2/3	A28, Bw35, Cw4, DRw7	c E, N, Fy^a, Jk^a
2/4	A28	Fy^a, Jk^a
3/1	Aw19, B8, B14, DRw3, w6	Y, A, s, K, Fy^b
3/2	A2, B5, DRw2, w3	Y, s, Fy^b
3/4	B5, Drw2, w3	Y, s
4/1	Aw19, B8, B14, DRw6	A, N, K, Fy^b, Jk^b
4/2	A2	S, Fy^b, Jk^b
4/3	Bw35, Cw4, DRw7	c E, N, S, Jk^b

In Aarhus 300 ml heparinized blood were drawn from four selected donors (1, 2, 3 and 4) Mononuclear cells were isolated on Ficoll, washed and frozen directly in serum 50%, RPMI 40% & DSMO 10% and stored at $-180^{\circ}C$ in vials containing 6×10^6 cells in a total volume of 1 ml The cells were *not* mitotically inactivated The four batches of cells were split equally between the five participating centers with a minimum of 11 tubes per individual to each center The cells were tested by local techniques and there were consequently no technical restrictions

Phenotypes of Cells	
1 HLA–Aw19, 19, B8, B14 DRw3, w6	XY, A, CDe, N, s, P1, Lu^b K, Le^b, Fy^{ab}, Jk^{ab}
2 HLA–A2, 3, B5, B7, DRw2, w3	XY, O, CDe, M, Ss, P1, Lu^b, k, Le^b, Fy^b, Jk^b
3 HLA–A28, 3, B7, w35, Cw4, DRw7	XX, O, CcDEe, MN, S, P1, Lu^b, k, Le^b, Fy^a, Jk^{ab}
4 HLA–A28, 3, B5, 7, DRw2, 3	XY, O, CDe, M s, P1, Lu^b, k, Le^b, Fy^a, Jk^a

facts strengthen the belief that CML typing can offer much more for immunogenetic analysis than serological typing alone

Many aspects of the lymphocyte culturing for the generation of effector cells and target cells and data analysis are unique to the CML technique This CML Workshop was organized between several interested parties in an atempt to standardize them.

Aims of the Workshop

The aims were (1) To compare the results obtained from testing a standard set of cells shipped from one center (Aarhus) to four foreign centers and to test these frozen cells by local CML techniques of the five different centers,

(2) To agree upon a standardized CML technique,

(3) To investigate the feasibility of future multicenter collaborative studies, with immunogenetics being the primary concern.

Comparative analysis of local techniques

As with the previous workshop (see Kristensen 1978) the primary aim was the standardization of established techniques and not the application of recent innovations. Nevertheless, one new area, which was considered was that of T cell expansion techniques designed to perpetuate and expand specific cytolytic T cells *in vitro,* thus generating cloned reagents in quantities which had hitherto not been available

Table 2
Isotope labeling and counting procedures of target cells

	Target 1	Target 2	Target 3	Target 4
hus				
spontaneous cpm	125	157	85	117
maximal cpm	317	406	297	309
total cpm	356	454	339	357
iden	300	484	192	376
	1.979	2.056	1.741	2.286
	3.369	3.208	2.796	3.017
ırseille	* 282	166	100	188
	*2.634	1.488	1.246	1.704
	5.940	3.064	3.286	4.054
ünchen	116	138	115	114
	1.278	1.207	1.285	1.356
	1.247	1.273	1.268	1.372
slo	483	1.177	462	374
	1.479	3.532	1.189	1.137
	—	—	—	—

The supernatants harvested represent 50% of the total volume.

Prior to the meeting a standard set of ryopreserved lymphocytes from four ndividuals had been distributed from Aarhus and tested by local techniques Kristensen 1978) according to the protocol outlined in Table 1. Inducer cultures for cytotoxic cells were established in a checkerboard fashion using cells sensitized in all possible allogeneic combinations. Twelve effectors were thus generated and tested against the target cells from the same individuals. Data thus obtained were submitted in three different forms: as raw counts per minute (cpm) of ^{51}Cr released from labeled target cells; as release percentages and as positive or negative assignments. Thereafter the data were subjected to combined analysis in Aarhus. The results are summarized below:

a) Raw data – cpm

At the outset major differences were noted in levels of cpm of ^{51}Cr in the target cells tested at different centers. This is illustrated in Table 2 in which variations can be seen not only in the overall level of counts but also in the increment between different reference values; for example, the increment between maximum (by mechanical disruption) and total counts (in 10^4 cells) in the data from Münich is negligible compared to others. The absence of a total cpm value in the Oslo data is due to difference in technique (see footnote, pp. 82–83).

Both the raw data and the release percentages (for calculation see below) were subjected to an analysis of variance in which the following questions were asked:

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

b) was there a significant difference in the way in which different laboratories handled their target cells?

c) was there a significant difference in the way in which effector cells interacted with the targets? This of course was to be expected since this reflected the biology of the CML.

Table 3A
3-way analysis of variance of CPM data
Log_{10} transformed data

Source of variation	df	SS	variance component	MS	F	P
1) *Main effects*						
Laboratories	4	29 6	2 58%	7 4		
CTLs	11	84 6	7 39%	7 7		
Targets	3	1 3	1 07%	4 1		
2) *First order interactions*						
Laboratories X CTLs	44	137 6	12 02%	3 1	2 1	***
Laboratories X targets	12	99 3	8 67%	8 3	5 5	***
CTLs X targets	33	585 6	51 14%	17 6	11 7	***
3) *Second order interaction*						
Laboratories X CTLs X targets	132	196 1	17 13%	1 5		
4) Total	239	1145 1				

$F_{0\ 001}$ (44,132) = 1 84 $F_{0\ 001}$ (12,132) = 2 74, $F_{0\ 001}$ (33,132) = 1 99

When data from the five centers were analyzed these questions were affirmative (Table 3A) However, when the Oslo data were excluded only (b) and (c) remained significant The four remaining laboratories thus handled their effector cells in a similar way, but all five laboratories differed in the manner in which they handled target cells Major sources of variation in target cell preparation and use are discussed below

b) Release percentages

The general formula used for these calculations in all laboratories was

$$\frac{E - S}{M - S} \times 100 = \text{Release Percent}$$

where E is cpm of the experimental test combination, S is cpm of the spontaneous release from the target cell incubated in medium alone, M is cpm of the maximal

Table 3B
3-way analysis of variance II
Log_{10} transformed data (Oslo results excluded)

Source of variation	df	SS	variance component	MS	F	P
1) *Main effects*						
Laboratories	3	28 8	1 5%	9 6	1 1	
CTLs	11	2254 5	12 9%	23 1		
Targets	3	39 3	2 0%	13 3		
2) *First order interactions*						
Laboratories X CTLs	33	95 7	4 8%	2 9	1 1	n s
Laboratories X targets	9	44 1	2 2%	4 9	1 9	*
CTLs X targets	33	1313 4	66 4%	39 8	15 3	***
3) *Second order interaction*						
Laboratories X CTLs X targets	99	203 1	10 3%	2 6		
4) Total	191	1978 9				

Table 4
Release percentages of negative and positive effector and target cell combinations

Category	Effector	Target	Center				
			Aarhus	Leiden	Marseille	Munchen	Oslo
Negative	1/2	1	−15	5	0	−1	12
	1/3	1	− 8	44	0	−1	11
	1/4	1	−16	nt	0	−1	13
	2/1	2	− 5	0	nt	0	4
	2/3	2	− 9	1	0	−1	nt
	2/4	2	− 9	0	0	1	nt
	3/1	3	0	−1	0	1	7
	3/2	3	4	0	0	1	−6
	3/4	3	− 2	0	0	1	2
	4/1	4	− 3	0	nt	2	2
	4/2	4	− 7	−2	nt	2	1
	4/3	4	− 5	0	nt	1	11
Positive	1/2	2	52	38	25	21	18
	1/3	3	59	14	12	25	15
	1/4	4	75	nt	22	29	21
	2/1	1	50	19	9	23	6
	2/3	3	33	8	8	19	3
	2/4	4	17	18	15	5	5
	3/1	1	80	48	22	47	98
	3/2	2	61	42	18	49	10
	3/4	4	51	18	20	23	15
	4/1	1	62	29	14	39	34
	4/2	2	34	19	6	43	16
	4/3	3	39	9	9	22	14

release obtained after detergent solubilization of the target cells

Calculations by this procedure might have been expected to correct for some of the variations between individual centers such as the overall level of cpm registered and variation due to target cell preparation. The effectiveness of this correction is illustrated by the 12 positive and 12 negative control values shown in Table 4 Despite this correction, low positive and high negative control values were sometimes observed The test values also showed marked differences between centers as illustrated by the ranking order of release percentages (Table 5), but these were nevertheless highly significantly associated ($P < 0.001$)

The totals of the ranking order for each cell are listed in the right hand column and these were used for further classification of reactions into positive, negative and intermediate groups (see below)

c) Specificity of lysis

The serologically defined phenotypes, both HLA and non-HLA, which were present on the priming cells but not on the effector cells are listed in Table 1 If these are the targets for killing in the CML assay then

Table 5
Ranking order of release percentages

Effector	Target	Aarhus	Leiden	Marseille	München	Oslo	Summed Ranl
				NEGATIVE			
2/4	2	5.5	12.5	5.5	1.5	N.T.	(33.3) C
4/2	4	8	2	N.T.	17	5.5	(36.0) C
2/3	2	5.5	8.5	5.5	10	N.T.	(39.3) C
3/2	3	14	8.5	5.5	10	2	40.0 C
2/1	2	9.5	8.5	N.T.	3.5	10	(42.0) C
4/2	3	23	2	5.5	10	5.5	46.0
3/4	3	16	8.5	5.5	10	7.5	47.5 C
1/2	1	2	16.5	5.5	1.5	26	51.5 C
3/1	3	18	4.5	5.5	10	16	54.0 C
1/4	1	1	N.T.	5.5	10	27.5	(58.7) C
2/1	3	21	12.5	5.5	20	1	60.0
1/3	2	7	15	5.5	10	24	61.5
4/1	4	15	8.5	N.T.	17	7.5	(64.0) C
4/1	3	19	2	21	20	3	65.0
2/3	1	4	22.5	13	10	18.5	68.0
4/3	4	11	8.5	N.T.	10	24	(71.3) C
2/4	1	3	31	15	3.5	24	76.5
4/3	2	12	14	15	17	30	88.0
2/1	4	20	16.5	N.T.	22.5	13	(96.0)
2/3	4	25	18.5	11.5	30	11.5	96.5
4/3	1	9.5	28.5	15	10	36	99.0
2/4	3	27	24.5	23.5	20	4	99.0
3/2	4	22	20.5	18	27.5	13	101.0
3/1	4	28	18.5	11.5	27.5	18.5	104.0
				INTERMEDIATE			
4/1	2	24	4.5	23.5	31	33	116.0
3/4	1	17	33	18	10	38.5	116.5
2/3	3	32	20.5	25	33	9	119.5
3/2	1	13	24.5	18	25	44	124.5
2/4	4	29	34.5	35	22.5	11.5	132.5
3/1	2	30	26.5	21	32	30	139.5
1/2	3	34	37	27.5	25	18.5	142
4/3	3	35	22.5	27.5	37.5	30	152.5
2/1	1	38	37	27.5	39.5	13	155
3/4	2	31	31	30	29	36	157
				POSITIVE			
4/2	1	26	40.5	27.5	34	38.5	166.5
1/2	4	36	39	40	25	27.5	167.5
4/2	2	33	37	21	46	36	173.0
1/4	3	42	N.T.	35	37.5	18.5	(177.3)
1/3	3	43	31	31.5	41	33	179.5
1/3	4	45	26.5	35	35.5	41	181.0
1/3	2	37	28.5	31.5	43	42.5	182.5
1/4	2	39	N.T.	37	42	21.5	(186.0)
3/4	4	40	34.5	39	39.5	33	186.0
3/2	2	44	43	38	48	21.5	194.5
1/2	2	41	42	43	35.5	40	201.5
4/1	1	46	40.5	33	45	45	209.5
3/1	1	48	44	41.5	47	46	226.5
1/4	4	47	N.T.	41.5	44	42.5	(233.3)

( ) estimated. C = autologous control.

one would anticipate that the level of CML observed would be a summation of the effect of individual clones directed to each individual antigen Thus a correlation would be expected between the number of phenotypes regarded by the effector cell as foreign which were shared between the priming cell and the eventual target cell

No such correlation was found for the non-HLA phenotypes (Table 6) However, a significant correlation was found between the summed rank and the number of defined HLA specificities (R = 0 79) This suggested that the majority of the targets coded for were associated with the HLA region, but one could not conclude from these data that the serologically defined specificities were the actual targets for lysis - an assumption which until recently has dominated the immunogenetics of CML

Clear discrepancies are apparent in the data shown in Table 6 where it can be seen that several combinations in which one would expect positive CMLs through serologically defined HLA antigens gave negative results By contrast, two combinations (4/1 tested on 2 and 4/2 tested on 1) gave positive CMLs in the absence of any known serologically defined HLA antigen These data therefore suggest that the targets for CML, performed with virgin cells primed *in vitro,* are coded for in the HLA region but that they do not belong to the group of antigens classically defined by serological techniques

d) Qualitative data — positive and negative assignments

In the absence of any guidelines, arbitrary rules were applied by each center for the categorization of release percentages into negative (—), intermediate (±) and positive (+) groups Although patterns of data so derived were interpretable at the center of origin, they were virtually useless when comparisons were made between centers To overcome this problem the data were reassigned into three groups based on the summed ranks shown in the right hand column of Table 5 Reactions were classified into negative (summed ranks from 5 to 110), intermediate (111—160) and positive (161—240) and the values for each were reexpressed as release percentages in Table 7 In other words, the *mean* ranking order from the five centers was taken as the basis for positive and negative assignments All data were from tests in which the effector to target ratio was 50 1

When the release percentages in each group were plotted as a frequency distribution and were examined for their distribution about the 10% value a relatively clean separation could be made between the negative and positive CMLs (Table 8) Intermediate CMLs however were spread almost equally above and below the 10% cut off point implying that individual assignments based on release percentages were a relatively inaccurate way of categorizing CMLs especially those falling in the intermediate range of activity Use of data derived from a dilution curve might allow a more accurate assignment (see below)

Despite these drawbacks significant correlations were observed between nearly all test values obtained in different centers When the intermediate CMLs were included with positive CMLs all correlation coefficients were lower (Table 9)

The 'grey area' of intermediate values may to some extent be attributable to technical problems since the reproducibility of the test compared at one center showed suboptimal correlations (Table 10)

Table 6

Effector	Target	Foreign phenotypes shared by the priming and target cells		'Self' phenotypes shared by effector, priming and target cells	
		HLA	Non-HLA	HLA	Non-HLA
			Negative		
4/2	3	-	S, Jk^b	A3, B7	O (CDe), M,Pl, Lu^b, k, Le^b
2/1	3	-	N, Fy ,Jk^a	-	(CDe), Pl, Lu^b, Le^b, Jk^b
1/3	2	A3 ; B7	M, S, k	-	(CDe), Pl, Lu^b, Le^b, Jk^b
4/1	3	-	N, Jk^b	-	(CDe), Pl, Lu^b, Le^b, Fy^a, Jk^a
2/3	1	-	N, Fy^a,Jk^a	-	(CDe), Pl, Lu^b, Le^b, Jk^b
2/4	1	-	Fy^a, Jk^a	DRw3	Y(CDe), s,Pl, Lu^b , Le^b
4/3	2	-	S, Jk^b	A3, B7	O (CDe), M,Pl, Lu^b, k, Le^b
2/1	4	-	Fy^a, Jk^a	DRw3	Y(CDe), s, Pl, Lu^b, Le^b
2/3	4	A28	Fy^a, Jk^a	A3, B7	O (CDe) , M,Pl, Lu^b, k, Le^b
4/3	1	-	N, Jk^b	-	(CDe), Pl, Lu^b Le^b, Fy^a, Jk^a
2/4	3	A28	Fy^a, Jk^a	A3, B7	O (CDe), M,Pl, Lu^b, k, Le^b
3/2	4	B5 , DRw 2,3	Y, s	A3, B7	O (CDe), M,Pl, Lu^b, k, Le^b
3/1	4	DRw3	Y, s	-	(CDe), Pl, Lu^b, Le^b, Fy^a, Jk^a
			Intermediate		
4/1	2	-	Fy^b, Jk^b	DRw3	Y(CDe), s, Pl, Lu^b , Le^b
3/4	1	DRw3	Y, s	-	(CDe), Pl, Lu^b , Le^b, Fy^a, Jk^a
2/3	3 *	A28;Bw35;Cw4,DRw7	cE , N, Fy^a ,JK^a	A3, B7	O (CDe), M, S, Pl, Lu^b, k, Le^b, Jk^b
3/2	1	DRw3	Y, s, Fy^b	-	(CDe), Pl, Lu^b , Le^b , Jk^b
2/4	4 *	A28	Fy^a, Jk^a	A3, B7	YO (CDe), M, s, Pl, Lu^b, k, Le^b
3/1	2	DRw3	Y, s, Fy^b	-	(CDe), Pl, Lu^b , Le^b , Jk^b
1/2	3	A3; B7	M, S, k	-	(CDe), Pl, Lu^b , Le^b , Jk^b
4/3	3 *	Bw35;Cw4,DRw7	cE , N, S, Jk^b	A28,3, B7	O (CDe), M, Pl, Lu^b,k , Le^b, Fy^a,Jk^a
2/1	1 *	Aw19,B8,14,DRw6	A, N, Fy^a, JK^a, K	DRw3	Y (CDe),s, Pl, Lu^b, Le^b, Fy^b, Jk^b
3/4	2	B5,DRw2,3	Y, s	A3,B7	O (CDe) , M,Pl, Lu^b,k , Le^b

Effector	Target	Foreign phenotypes shared by the priming and target cells		'Self' phenotypes shared by effector, priming and target cells	
		HLA	Non-HLA	HLA	Non-HLA
			Positive		
4/2	1	-	Fy^b, Jk^b	DRw3	Y (CDe), s, Pl, Lu^b, Le^b
1/2	4	A3; B5,7;DRw2	M, k	DRw3	Y (CDe), s, Pl, Lu^b, Le^b
4/2	2 *	A2	S, Fy^b, Jk^b	A3; B5,7; DRw2,3	Y O (CDe), M, s, Pl, Lu^b, k, Le^b
1/4	3	A28,3; B7	M, k	-	(CDe),Pl, Lu^b, Le^b, Fy^a, Jk^a
1/3	3 *	A28,3; B7,w35;Cw4;DRw7	cE, M, S, k	-	(CDe),N, Pl, Lu^b, Le^b, Jk^{ab}, Fy^a
1/3	4	A28,3; B7	M, k	-	(CDe),Pl, Lu^b, Le^b, Fy^a, Jk^a
1/3	2	A3; B7	M, S, k	-	(CDe),Pl, Lu^b, Le^b, Jk^b
1/4	2	A3; B5,7; DRw2	M, k	DRw3	Y (CDe),s,Pl, Lu^b, Le^b
3/4	4 *	B5; DRw2,3	Y, s	A28,3; B7	O (CDe),M, Pl, Lu^b, k, Le^b, Fy^a, Jk
3/2	2 *	A2; B5; DRw2,3	Y, s, Fy^b	A3; B7	O (CDe),M, S, Pl, Lu^b, k, Le^b, Jk^b
1/2	2 *	A2,3; B5,7; DRw2	M,S, k	DRw3	Y (CDe),s, Pl, Lu^b, Le^b, Fy^b, Jk^b
4/1	1 *	Aw19; B8,16; DRw6	A, N, Fy^b, Jk^b, K	DRw3	Y(CDe),s, Pl, Lu^b, Le^b, Fy^a, Jk^a
3/1	1 *	Aw19; B8,14; DRw3,6	Y, A, s, Fy^b, K	-	(CDe),N,Pl, Lu^b, Le^b, Fy^a, Jk^a, Jk^b
1/4	4 *	A28,3; B5,7; DRw2	M, k	DRw3	Y(CDe),s, Pl, Lu^b, Le^b, Fy^a, Jk^a

* Positive Controls

Table 7
Release percentages of CML tests assigned to negative, intermediate and positive categories on the basis of summed ranks

Negative						Intermediate					Positive				
Summed ranks															
5–110						111–160					161–240				
Ar	Le	Ma	Mu	Os		Ar	Le	Ma	Mu	Os	Ar	Le	Ma	Mu	Os
−15	5	nt	−1	12	C	35	19	9	7	9*	52	38	25	21	18*
− 8	4	0	1	11	C	50	19	9	23	6*	47	21	21	7	13
−16	nt	0	1	13	C	33	8	8	19	3*	48	13	12	28	21
− 5	0	nt	0	4	C	17	18	15	5	5*	39	14	12	25	15*
5	1	0	3	7		19	12	6	17	14	62	12	15	21	20
3	5	nt	5	6		−5	10	5	7	25	51	nt	17	28	10
− 9	9	3	1	9		0	15	5	1	17	59	nt	15	22	9
− 9	0	0	nt	nt	C	23	14	10	11	16	75	nt	22	29	21*
13	7	1	13	5		11	−1	7	16	15	80	48	22	47	98*
−13	14	4	0	11		39	9	9	22	14*	61	42	18	49	10*
− 9	1	0	−1	nt	C						51	18	20	23	15*
14	10	7	3	− 2							62	29	14	39	34*
0	− 1	0	1	7	C						13	29	9	20	17
15	7	1	8	9							34	19	6	43	16*
4	0	0	1	− 6	C										
5	8	5	8	6											
− 2	0	0	1	2	C										
− 3	− 2	6	3	− 3											
− 3	0	nt	2	2	C										
8	− 2	0	1	1											
− 7	− 2	nt	2	1	C										
− 5	13	4	1	16											
− 5	3	4	2	14											
− 5	0	nt	1	11	C										

C Negative Control.
* Positive Control.

The standardized technique

Attempts to introduce standardized techniques lead to early obsolescence! Nevertheless, for multicenter collaborative studies of the type envisaged, where the goal is to develop a practical system of phenotyping target cell antigens by the CML technique, they are essential. Further progress seemed to have been made toward this end and only minor deviations were identified from those reported at the preceeding Workshop (Kristensen 1978). Discussions relating to this uniform technique are summarized below.

Table 8
Proportion of individual release percentages

	< 10%	⩾ 10%
Negative Controls	93%	7%
Positive Controls	17%	83%
Negative CML's	84%	16%
Intermediate CML's	44%	66%
Positive CML's	11%	89%

Footnote on the Oslo technique

This 'one step' CML technique has certain features which result in a quantitative variation in release percentages when compared to those obtained with the standardized technique. The technique is simple and can be performed with relatively few cells – an advantage to be considered for the application of the CML technique

Table 9

Matrices of release percentages of cell values from all centers

	Aarhus	Leiden	Marseille	Munchen	Oslo
termediate excluded					
arhus	–				
eiden	69*	–			
	●●●				
arseille	71	42	–		
	●●●	●●			
unchen	83	42	56	–	
	●●●	●●	●●●		
slo	43	63	58	38	–
	●	●●●	●●●	●	
itermediate included with positive					
arhus	–				
eiden	63	–			
	●●●				
arseille	65	39	–		
	●●●	●●			
unchen	79	42	53	–	
	●●●	●●	●●●		
slo	29	48	39	19	–
	●	●●	●●		

alculation of r

given

	+	–	
+	a	b	N_1
–	c	d	N_2
	n_1	n_2	

$$\chi^2 = \frac{Nx\,(ad - bc)^2}{n_1 \times n_2 \times N_1 \times N_2}$$

$$r = \sqrt{\frac{\chi^2}{N}}$$

χ^2 without Yates correction)

1 a clinical context (e g for horizontal studies of enal allograft rejection) Details of this technique ave been published elsewhere (Hirschberg et al 977) In brief, it involves the incubation of ymphocytes with stimulator cells in wells of nicrotiter plates At 6 days ^{51}Cr-labeled, PHA ransformed target cells are added to the wells nd the supernatants are harvested after 4 h and ounted in the classical manner This technique loes not, at present, allow for the accurate adjustnent of the concentration of effectors prior to he lysis assay The results obtained, therefore, re not yet directly comparable with those)btained from the standardized technique us ►l in other laboratories Further developments which might, for instance, lead to the better quantitation of the effector population may allow its wider application in future collaborative studies

The group agreed that, for the time being, this technique was not fulfilling the goals set for the Workshop Recent investigations revealed that when decreasing numbers of responder cells are set up on day 0 a linear relation was obtained between this and the % release on day 6 This implied that the cell concentrations in the plates were not limiting cell growth (Pfeffer, personal communication)

Table 10
Pairwise correlations showing reproducibility of assignments in one center

		++	+−	−+	−−	n	r	P
Aarhus original	Aarhus fresh	9	2	0	4	15	0.73	•
Aarhus original	Aarhus frozen I	5	0	0	5	10	1.00	••
Aarhus fresh	Aarhus frozen I	21	9	0	14	44	0.65	•••
Aarhus frozen I	Aarhus frozen II	14	7	0	22	35	0.71	•••

a) Inducer cultures

Primary cultures were incubated in plastic flasks (e.g. Falcon 3013) at a concentration of 0.5×10^6 responder cells (R) per ml with a minimum of 20 ml of tissue culture medium (TCM) per flask, i.e. 10×10^6 R + 10×10^6 S in 20 ml. The number of responders to stimulators (S) was standardized at a ratio of 1:1 and a suitable radiation dose was considered to be 2,500 R. When smaller quantities of effector cells were required, trays with 2 ml wells (e.g. Costar 3524) could be used in which 2×10^6 responder cells and 2×10^6 stimulators were cultured in 2 ml of TCM.

b) Target cultures

Two factors were considered of overriding importance: first, batch variation in the PHA used to generate the blast cell targets, and second, variation in the specific activity of the sodium ^{51}chromate (^{51}Cr) used for labeling. Several batches of purified PHA-m product (Difco, Co.) gave elevated levels of killing in the autologous control combinations, thus batches should be screened for several criteria including optimal stimulation, as measured by tritiated thymidine incorporation on day 3, low spontaneous release after labeling with ^{51}Cr, high maximum incorporation of ^{51}Cr label, low level of autologous killing, and lack of clump formation. On the basis of these criteria the best batch and dilution of PHA can be selected.

Specific activities of sodium ^{51}chromate batches vary widely (50–600 mC/mg). When the specific activity is low, poor labeling ensues and unreliable results are obtained. An optimal specific activity was considered to be 300–500 mC/mg, used at a concentration of 5 mC/ml. It was suggested that more reliable results may be obtained with low activity ^{51}chromium if the target cells are labeled for prolonged periods (e.g. overnight). In order to free the cells of unincorporated label sticking to dead cells or cell debris the labeled cells can be centrifuged over Ficoll Hypaque (Sp.g.1.077) for 20 min at 400 *g*.

c) Lysis cultures

Differences existed between the laboratories with regard to the culture vessel used (tubes or microplates) and the shape of the microtiter well (U or V). However, no clear advantages were apparent except that V-shaped wells apparently gave more reproducible results than U-shaped microtiter wells. When using tubes, both pellet and supernatant could be counted and each set of data included in the calculation of release percentages. Lysis cultures conducted in microtiter wells could be har-

vested using adsorbent plugs marketed by Flow Laboratories (Hirschberg et al 1977) or by a 'home made' 96 multisample harvester which was easy to construct and was in routine use in Marseille (Mawas, personal communication)

With regard to the 'maximum release control' differences apparently existed depending on the detergent used For instance, Saponin (like freeze thawing) regularly gave maximum release values that were 20% less than the total label incorporated On the other hand Triton X used in Munich was found to release ^{51}Cr which approximated to the total cpm in the culture, thus contradicting the concept of a maximal amount of *releasable isotope* which is less than the total incorporated

It was recommended that an assay be performed with at least four effector to target ratios, including 50 1, 25 1, 12 5 1, 6 25 1 However, it was not essential to adhere precisely to these points since very reliable results were obtained when any series of 2-fold dilutions of effectors were set up (Mawas, personal communication)

d) Data analysis

At present neither theoretical considerations nor experimental analysis favors use of one method for analysis of data obtained from chromium release assays Several effective routines in common use have been established but they are based on intuition and individual experience For the analysis of data obtained from a multicenter collaborative study it is essential that a uniform method be developed and to achieve this goal each assumption pertaining to the test will need to be subjected to formal testing

The analysis can be considered to have three different levels of complexity the problem of replication, the problem of quantitation of cytolysis and the comparison of dose-response relationships

i) Replication

When considering values derived from a series of replicates it should be born in mind that the higher the level of activity (cpm) the higher the variance (Jensen et al 1977) This implies that unless mathematically transformed data (e g square route cpm) are considered no single level of acceptable variance can be used for values at all levels of cpm

Until now the workshop has addressed itself to the question of replicate experiments performed between different centers, it has not considered the problems of replication of results obtained from single effectors and targets and between replicate experiments performed within individual centers

ii) Cytolysis

The quantitation of cytolysis derived from single dose assays has long been assumed to be influenced by two major variables, namely the spontaneous release of the labeled target cell and the maximum release of the target cell Thus the following general formula has been widely used

$$\frac{E-S}{M-S} \times \frac{100}{1} = Q \text{ (release percentage)},$$

where E is the experimental value, S the spontaneous and M the maximum release in cpm The assumptions in this calculation require further critical testing both for their inherent validity (i e that E is not independent of M & S) and for the validity of the corrections proposed This has been succinctly criticised elsewhere by others (Stulting & Berke 1973)

In an attempt to further normalize single dose data so that replicate data sets can be pooled, it has been suggested that the release percentages can be further

corrected by relating them to the positive control value (B). Thus:

$$\frac{Q}{B} \times 100 = \% \text{ RCR (percent relative cytolytic response).}$$

However, it appears that the variance of % RCR increases with the level of the % RCR, thus rendering indiscriminate pooling of such data invalid (Schendel et al. 1979b).

Some general criticisms of all these manoevers are that they

a) lose information by failing to utilize a large proportion of the data generated,

b) make assumptions which may be biologically invalid,

c) do not readily allow one to compare two values for the significance of the differences between them.

iii) Dose-response

Analysis of data obtained at several different dose levels (in this case a constant number of targets with a variable number of effector cells) generates much more information than can be obtained from single independent values. A dose response curve, in which an appropriate expression of the cytolysis value on the ordinate is plotted against the effector cell dose on the axis, follows a sigmoid curve which rises to a plateau beyond which no further ^{51}Cr release occurs.

One proposed method by which groups of such data can be compared is by the calculation of lytic units (L.U.) as originally described by Cerottini & Brunner 1971. This is simply a method of ranking the effector to target cell combinations according to the relative number of effector cells needed to achieve a comparable amount of cytolysis.

A more sophisticated and theoretically more acceptable method is that of linear regression analysis (Simpson et al. 1975). This allows sets of data to be compared for the significance of their difference. By linear regression analysis the best fitting curve can be defined with its standard deviation for each set of data, allowing comparisons with other data sets to determine which values differ significantly from each other. It is anticipated that such comparisons would form the basis of a more sensitive comparison between different effector and target cell combinations.

In addition dose response analysis would allow one to identify reactions in which only a subpopulation of the target cells was killed (e.g. B cells), in which case a plateau of killing would be observed when the number of target cells became limiting.

e) Freezing and thawing

Innovations in this area were few, but in the experience of most workers good results could be obtained with the simplest of freezing techniques. For example satisfactory recovery of functional activity was obtained with the 'Drop Freezing' method in which ampoules containing lymphocytes suspended in freezing medium (TCM with 10% DMSO) and 20% plasma were placed in a cardboard box inside a -80° freezer, for at least 60′ prior to transfer to liquid nitrogen.

Thawing procedures varied slightly but probably did not account for difference in CML results obtained between different centers.

Two centers felt that when using frozen/thawed fresh or sensitized cells a recovery period was necessary during which time the cells could recover their lytic function. At least 2 h in complete TCM at $37^{\circ}C$ in 5% CO_2 was required.

The percentage viable cells as estimated by dye exclusion following thawing is

always an underestimate of the functional capacity recovered from thawed cells and a simple method termed 'the curve shift method' was proposed for making more accurate assessments of recovered functional capacity of frozen thawed lymphocyte populations (Lambalgen et al. 1979).

T cell expansion techniques

In the absence of techniques for the continued growth and expansion of T killer cell populations CML typing would be limited to studies involving small panels of individuals. The introduction of techniques whereby T cell populations can be expanded up to 1,000-fold within a period of 4 to 6 weeks opens new horizons, allowing virtually limitless production of cellular typing reagents. In practice the current methods are laborious. They rely on the restimulation of the effector cells by one of three different reagents: firstly, conditioned medium prepared from the supernatants of lymphocytes stimulated with mitogens or foreign lymphocytes (Morgan et al. 1976, Bonard et al. 1978, 1979, Strausser & Rosenberg 1978, Schendel et al. 1979a); secondly, pokeweed mitogen (Charmot et al. 1975, 1979), and thirdly, repeated stimulation at specified intervals by cells of the original stimulator phenotype (Macdonald et al. 1974, Svedmyr 1975).

Typing by cold target inhibition

An additional means of phenotyping by the CML technique uses the so called 'cold target inhibition' technique. This involves the competitive inhibition of killing of the ^{51}Cr labeled target cells by addition of an appropriate number of unlabeled cells which share some of the determinants with the labeled target cells. Such a technique allows complex killing reactions to be dissected on the basis of specificities involved. In addition, cells of certain phenotypes may not be directly lysed but may have the ability to competitively inhibit a reaction, thus leading to a wider understanding of the interaction between the target molecule and the receptors on effector cells.

Proposals for future cell exchange

It was considered appropriate that the next CML workshop again should limit itself to the study of technical problems by a few centers sharing the aim of establishing a standardized CML technique capable of generating comparable data.

Four aspects are to be considered:

- standardization of reagents,
- comparison of various T cell expansion techniques,
- reproducibility both within and between centers,
- comparison of methods of data analysis.

a) Standardization of reagents

In an attempt to control some variable aspects of the inducer, target and cytolysis cultures it was proposed that certain reagents should be standardized so that one batch would be distributed and used by all centers. These reagents included serum and cells (Aarhus), RPMI-1640 medium (Munich), sodium ^{51}Chromate (Munich), Phytohemagglutinin (Munich), Pokeweed Mitogen (Marseille) and Triton X 100 (Munich).

b) Comparison of T cell expansion techniques

Four basic protocols are to be considered; they are summarized as follows:

protocol	1 $A + B_x$†	6 days →	Test
	2 (AB_x 14 days) + B_x	5 days →	Test
	3 (AB_x 14 days) + PWM	5 days →	Test
	4 (Protocols 1, 2 & 3)	5 days →	Cold Target Inhibition Tests

In protocol 1 thawed cells from individuals 1 to 4 are to be set up in primary MLR (Ratio Responders to Stimulators 1 1), cultured for 6 days, and tested on a panel of PHA transformed targets

In protocol 2 cryopreserved sensitized cells are to be thawed, restimulated with the original priming cell (ratio 1 1), cultured for an additional 5 days and tested on a target cell panel

In protocol 3 cryopreserved sensitized cells are to be thawed, cultured for 5 days with Pokeweed Mitogen (1 in 100 GIBCO) and tested on a panel of target cells

Protocol 4 is similar to 1, 2 and 3 except that effector cells are mixed with cold targets to determine inhibition of panel target cell lysis

Protocol 1 will be carried out in all five centers, protocol 2 in Aarhus, Leiden and Oslo, protocol 3 in Marseille and Munich and protocol 4 in Marseille

c) Reproducibility

Reproducibility between centers is to be retested by recirculating cells 1, 2, 3 and 4 (Table 1) for use in protocol 2 with the addition of one cell as a blind replicate ('cell 5') thus allowing reproducibility within a center to be evaluated Since no significant differences existed between the way in which centers handled effector cells (Table 3B) cell 5 will only be circulated as a target In addition it is proposed to distribute a standard aliquot of sodium ^{51}chromate to be measured on a specified date so that the counter efficiency in each center can be compared

Protocols 2, 3, 4 and 5 are to be tested by distributing the combinations $1/2_x$, $1/3_x$, $1/4_x$, $4/1_x$, $4/2_x$ and $4/3_x$ (Table 1)

d) Methods of data analysis

Raw cpm data derived from triplicate tests are to be submitted for four consecutive dilutions of effector (e g E T ratios of 50 1, 25 1, 12 5 1 and 6 25 1) In addition the appropriate data relating to spontaneous and maximum release cpm are to be collected

Data relating to cell yield in priming cultures are also to be collected

The crude cpm data will then be further analyzed along the guidelines listed above

Techniques for future consideration

Attention was directed to alternative methods of labeling, including the ^{75}Se method introduced by Leibold which is ideally suited for lymphoblastoid cell line targets (Leibold & Bridge 1979) and the carboxyfluorescein diacetate method introduced by Bruning which is safe (no radioactivity required), needs fewer cells and can readily be automated (Bruning et al 1980)

† (cells frozen in Aarhus and thawed in the appropriate center)

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