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CARBOXYFLUORESCEIN FLUOROCHROMASIA CELL-MEDIATED LYMPHOLYSIS A COMPARATIVE STUDY

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1. Introduction

A method for cell mediated lympholysis (CML) has been developed in which carboxyfluorescein substitutes the radioactive chromium label [1,2]. Here we report more extensively on the comparison of the results obtained in the fluorochromasia assay, a micro-CML assay read with an automated microfluorometer, and those of the [^{51}Cr] release assay. The standard deviations of the release percentages are smaller in the chromium assay than in the fluorochromasia (CF) assay. However, results obtained with both methods give identical positive and negative assignments. In our opinion the fluorochromasia CML is an attractive alternative for the chromium method and has some major advantages: (1) no radioactivity, (2) the requirement of fewer effector cells, (3) speed of procedure (short labeling time, simple wash procedures), (4) fast reading of results in an automated microfluorometer and (5) economy. We also describe some modifications of the original method which, apart from increasing the sensitivity of the assay, simplify even more the technique.

2. Materials and methods

2.1. *Effector cell preparation*

Effectors were generated in a one way mixed lymphocyte reaction (MLR) essentially as described by Goulmy [3]. Briefly, 10^7 responder and 10^7 irradiated stimulator lymphocytes (2000 Rad) were cultured for 6 days in a humidified 5% CO_2 incubator at 37°C in a 30 ml flask in 20 ml culture medium (RPMI 1640, 2 mM glutamine, 100 I.U./ml penicillin and 100 μg/ml streptomycin, supplemented with 20% pooled AB serum and 10 mM Hepes (final concentration)). Alternatively 10^6 responder and 10^6 stimulator cells were cultured in Costar macro well plates in 2 ml vols. After incubation cells were centrifuged at 350 × *g* for 10 min, the supernatant decanted and the effector cells resuspended in culture medium at the appropriate concentration.

2.2. *Target cell preparation*

Cells used to set up the MLR were cultured alone at a concentration of 10^6/ml in 10 ml of culture medium. On the third day 0.1 ml of phytohaemagglutinin (PHA M) Bacto-Difco was added, giving a final concentration of 1% PHA. On day 5, 8 ml of the supernatant medium was replaced by fresh medium. This was done to have no aggregates in the target cell suspension on day 6. The absence of aggregates is essential for reproducible dispensing of equal numbers of target cells in the wells of microtest plates for the fluorochromasia assay. For the chromium assay the medium was not refreshed on day 5.

2.3. *Labeling of target cells*

Target cells were collected by centrifugation for 10 min at 350 × *g* and washed twice with Hanks solution. Finally the cells were suspended in 1 ml freshly prepared carboxyfluorescein diacetate solution in RPMI (final concentration 40 μg/ml) and incubated for 15 min in a 37°C waterbath. After labeling the cells were washed once with RPMI and resuspended at a concentration of 8×10^5/ml in protein free RPMI (or alternatively complete medium, see Results

3 3) For the Cr release assay targets were mixed with 100 μCi $Na_2{}^{51}CrO_4$ and incubated for 1 h in a 37°C waterbath with shaking after 30 min. After labeling the targets were washed twice with Hanks and resuspended in culture medium at a concentration of 10^5/ml.

2.4 *Fluorochromasia assay*

4000 C.F. labeled target cells in 5 μl protein free Hanks solution were applied to each well of a Terasaki type microtest-tray with a Hamilton syringe. The trays were spun for 5 min at 350 × *g* for fixation of the targets. The wells were flooded with complete medium and after standing for 5 min with occasional mixing by tilting, the tray was decanted and gently flicked to remove most of the fluid from the wells. Positive control values were obtained by leaving at least one of the wells without target cells. To wells containing target cells effector cells or negative controls were added in 10 μl samples. Negative controls were heat treated effector cells incubated for 15 min in a 46°C waterbath. To avoid detachment of the fixed targets the samples were not injected into the wells but droplets were formed at the needle tip of the Hamilton syringe and applied by touching the well rim. The trays were then centrifuged for 2 min at 150 × *g* read in the automatic fluorometer and incubated in a humidified 5% CO_2 incubator at 37°C for 4 h. After incubation the trays were centrifuged at 400 × *g* for 5 min and flooded carefully with phosphate buffered saline (PBS) flowing freely from a syringe barrel with a 1 mm needle to elute released C.F. After removal of the saline by decantation the trays were read again in the automated fluorometer. The readings in one tray, which always included negative and positive control wells were normalized by the computer program in percentages. The fluorometer readings before and after incubation were recorded. The latter reading of each individual well was divided by the first reading. The ratios of replicate wells were averaged and used as a percentage for the calculation of the percentage release with the formula

$$\left(1 - \frac{\text{mean test}}{\text{mean control}}\right) \times 100$$

2.5 *Quenching of medium fluorescence*

Erythrocytes (human) were washed 3 times with PBS and the packed cells lyzed by freeze thawing. After removal of the cell debris by centrifugation for 10 min at 800 × *g* the hemoglobin solution was dialyzed against PBS for 48 h. The concentrated hemoglobin was stored in small vials at −20°C. At the end of the incubation period 5 μl of a 4 mM isotonic hemoglobin solution (clarified by centrifugation after dilution) was applied to each well by tipping off preformed droplets from the syringe needle(s). Diffusion was allowed for 10 min at room temperature after which the cells were packed by centrifugation at 400 × *g* for 5 min. The microtest trays were read in the automated fluorometer and the percentage release calculated as described in 2.4.

2.6 *Chromium release assay*

Effector cell suspensions (0.1 ml) and target cell suspensions (0.1 ml) were added to the wells of a microtiter tray using a Hamilton syringe. The trays were spun for 2 min at 150 × *g* and incubated for 4 h in a humidified 5% CO_2 incubator at 37°C. After incubation the trays were centrifuged for 5 min at 400 × *g*. Supernatants were harvested using a commercial supernatant harvester system and the samples counted in a γ counter Searle 1195. The counts were used to calculate percentage release using the formula

$$\frac{\text{experimental mean} - \text{spontaneous mean}}{\text{maximal mean} - \text{spontaneous mean}} \times 100$$

By plotting the per cent release value against the logarithm of the ratio of effector to target cells one lytic unit (LU) could arbitrarily be defined as the number of effector cells which lyze 30% of 10^4 target cells ($[^{51}Cr]$ CML) or 4×10^3 target cells (C.F. CML) within 4 h.

2.7 *Automated microfluorometer*

Inverted epi illumination fluorescence microscope (Leitz Diavert) equipped with an automated stage driven motor by stepping motors (Leitz) and a photomultiplier (Zeiss) encased in a black plastic box with counter balanced door. Digital Equipment Corporation PDP8/L computer (4K memory) high speed paper tape reader and punch and a Teleprint 303 writer. Light source is a 60 W tungsten lamp mounted outside the microscope box and fed from a stabilized

power supply Filters standard FITC filter mirror combination with 6 mm BG 38 filter in excitation light path Objective is a 6 3 × Neofluar

2 8 *Lymphocyte donors*

Healthy donors were used which were typed for HLA A, B C and DR antigens [4 5]

Part of the work shown here was done in the context of the Third European CMI Workshop held in December 1979 in Marseille [6 7]

Cells were selected frozen and shipped from Aarhus

2 9 *Statistical analysis*

Standard deviations of the release percentages were calculated for the chromium and the fluorochromasia assays using Formula 1 and 2 respectively

Formula 1

$$sd_{CR} = \left[(\text{variance (experimental cpm)})^2 + \left(\frac{\text{exp} - \text{max}}{\text{max} - \text{spon}}\right)^2 \times \text{variance } (\overline{\text{spon}} \text{ cpm}) + \left(\frac{\text{exp} - \text{spon}}{\text{max} - \text{spon}}\right)^2 \times \text{variance (max cpm)} \right]$$

Formula 2

$$sd_{CF} = \left[\left(\frac{\text{exp}}{\text{control}}\right)^2 \times \left(\text{variance } \frac{(\text{exp})}{\text{exp}} + \text{variance } \frac{(\text{control})}{\text{control}} \right) \right]^2$$

$$\frac{\text{exp}}{\text{control}} \left((\text{variation coefficient exp})^2 + (\text{variation coefficient control})^2 \right)^2$$

3 Results and discussion

3 1 *Comparison of the fluorochromasia and chromium assay*

In order to assess whether the fluorochromasia assay and the chromium assay give identical results effector cells were made between members of family F (Table 1)

Sib C2 and C4 which are HLA A B C and DR identical and MLC negative (data not shown) were tested both for responder and stimulator capacity The effectors used for the comparison test came from one stock suspension and were tested in both assays on the same day The results expressed in lytic units (Table 2) show a good concordance between both techniques

All cytotoxic reactions essentially paralleled each other in both assays There are some discrepant reactions however in particular the fit for target F is not complete This is probably due to the fact that the targets used in the comparison test, were handled slightly differently for both assays For the fluorochromasia assay the medium of the PHA cultures was replaced by fresh medium at day 5 while this was not done for the chromium assay Since frozen cells were used the blast transformation might not have been optimal for the targets in the fluorochromasia assay, resulting in a suboptimal target cell preparation Alternatively, the difference is due to the fact that the targets were taken from two different culture flasks The reactions on the other 5 targets were not disturbed by this procedure

The results of the CF CML and [^{51}Cr] CML can be compared in an other way, namely by plotting the specific release percentages in a correlation diagram This results in a cluster of points through which a regression line can be projected as shown in Fig 1 The regression line has a coefficient of determination $r = 0.89$ Ideally the coefficient of determination should equal 1 This demonstrates also the concordance of the specific release percentages in both assays

Our laboratory also participated in the third CML workshop which aimed at standardizing the CMI technique [6 7]

Table 1
Family 1

	Paternal		Maternal	
Haplotype	a	b	c	d
Father	A1 B8 Cw DRw6	Aw24 Bw44 Cw1 DRw2		
Mother			A3 B7 Cw DRw1	A1 B17 1 Cw6, DRw7
Child 1		Aw24 Bw44 Cw1 DRw2		A1 B17 1 Cw6 DRw7
Child 2		Aw24, Bw44 Cw1 DRw2	A3 B7 Cw DRw1	
Child 3	A1 B8 Cw DRw6		A3 B7 Cw DRw1	
Child 4		Aw24 Bw44 Cw1 DRw1	A3 B7 Cw, DRw1	

In brief the scheme of the workshop was such that all participating laboratories used the same cells distributed by one of the centers and used standardized reagents (medium serum and PHA). Six effector combinations were tested on 5 targets in primary and secondary CML using a standardized protocol [7].

The results of the fluorochromasia and chromium assay performed in the Leiden laboratory are shown in Fig. 2. The regression line projected has a coefficient of determination $r = 0.81$. The concordance of the cytotoxic reactions is very good, taking into account that the effector and target cells used came from different bulk cultures and were tested on separate days. For this reason the coefficient of determination, r, for the Workshop CML is somewhat lower than for the family CML.

3.2 Analysis of variance

To determine the variation in the specific release percentages obtained in the fluorochromasia and the chromium assay the standard deviations were calculated using the formulae shown in 2.8. It is clear that the chromium assay has a lower standard deviation (mean S.D. = 1.04) than the fluorochromasia assay (mean S.D. = 2.97) for the family CML. The workshop CML gives essentially the same result.

Table 2
Comparison of CL and [^{51}Cr] CML in Family 1

Effectors		Targets	M	I	C1	C2	C3	C4
C1 vs C2	^{51}Cr		2[a]	1.1	0	0.4	1.4	2.8
	CL		1	0.2	0	0.4	0.8	1.6
C1 vs C4	Cr		3	1.7	0	0.6	2.0	3.2
	CL		1	0.8	0	0.9	0.8	3.4
C2 vs M	^{51}Cr		9.2	2.2	8.7	1.1	1.1	0.3
	CL		7.7	0.5	4.6	0.8	1.4	0.1
C4 vs M	^{51}Cr		7.7	3.3	6.3	0.5	1.2	1.3
	CL		7.7	1.6	4.0	0.5	0.9	0.8
C3 vs M	Cr		4.6	0	3.3	0	0	0
	CL		5.3	0	2.8	0	0	0
C3 vs I	^{51}Cr		0	3	2	1.1	0	2.7
	CL		0	1.8	1.4	1.0	0	1.7

[a] The results are expressed as lytic units per 10^6 effector cells (see also section 2.5).

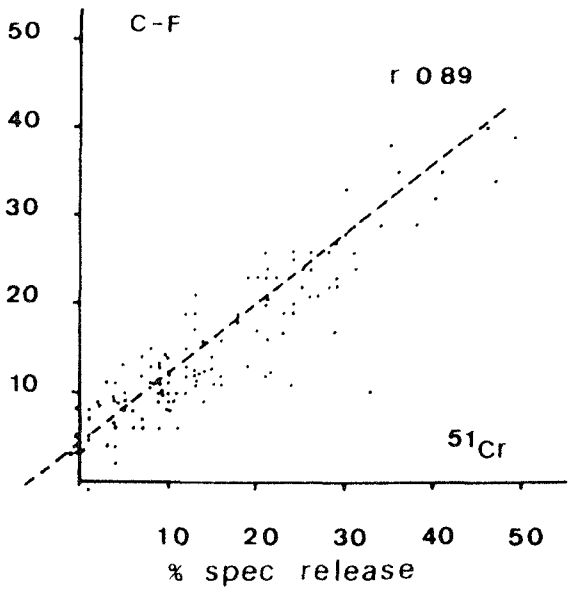

Fig. 1. Correlation diagram of release percentages in C-F and [^{51}Cr] CML of Family F. Each point represents the release percentages at one dilution (40, 20, 10 or 5:1 effector target ratio, respectively) for one effector on the corresponding target as measured in both assays. The regression line is projected through the cluster of points, r is the coefficient of determination.

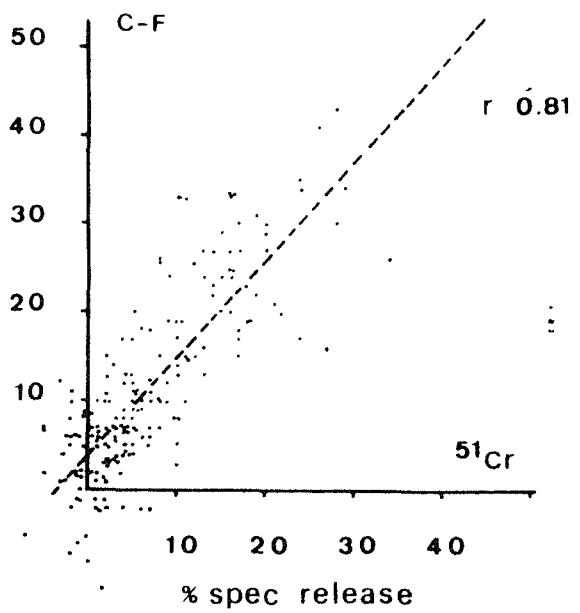

Fig. 2. Correlation diagram of release percentages in C-F and [^{51}Cr]-CML assays performed for the Third European CML Workshop. The results shown are performed in the Leiden group (see also legend for Fig. 1).

Table 3
2 × 2 Analysis of assignments

CML Workshop[a]	1° CML		++	+	−+	−−
C-F vs ^{51}Cr		Leiden	14	1	1	9
		Aarhus	17	2	0	11
		Marseille	14	3	1	7
		Munich	19	0	1	10
		Oslo	5	6	1	8
^{51}Cr Leiden vs ^{51}Cr		Aarhus	13	2	0	10
		Marseille	10	3	1	6
		Munich	15	0	1	9
		Oslo	6	5	0	9
^{51}Cr Aarhus vs ^{51}Cr		Marseille	13	3	2	7
		Munich	16	0	3	11
		Oslo	5	4	1	10
^{51}Cr Marseille vs ^{51}Cr		Munich	15	0	3	7
		Oslo	4	3	1	7
^{51}Cr Munich vs ^{51}Cr		Oslo	6	6	0	8
II Family F						
C-F vs ^{51}Cr			28	0	0	8

[a]Results based on assignments made in the Third European CML Workshop [7].

[^{51}Cr] CML, mean SD = 1.87 and CF CML mean SD = 3.87. Although the error in the percentage cytotoxicity is greater for the CF CML this is not reflected in the positive or negative assignments of the reactions (Table 3).

When comparing the data obtained in the Third European CML Workshop, the results of the CF CML cannot be distinguished from those of the chromium CML assays on the basis of the number of discrepancies.

Because of the excellent agreement of positive and negative reactions we feel the carboxyfluorescein CML is as reliable as the chromium release CML. Despite the greater standard deviation in the specific release percentage, the CF CML has some major advantages. No radioactivity is involved, while the carboxyfluorescein diacetate can be made easily from commercially available carboxyfluorescein [1]. About half the number of effector cells is required compared to the chromium assay. Labeling of targets takes less time and the washing procedures are simple. Together with the fast reading of the results in the automated microfluorometer (1 min/tray) the procedure is much faster than the chromium CML.

3.3. *Modifications of the fluorochromasia assay*

The most inconvenient part of the fluorochromasia assay is the fixation of target cells to the bottom of the wells in the tray. The procedure is relatively time consuming and laborious. Therefore it was attempted to use the same procedure as for the chromium assay i.e. simple mixing of effectors and targets in the wells without prior fixation of the targets. The results of a CML with targets fixed to the well bottoms and targets mixed with effectors is shown in Table 4. It is clear that the specific release is greater in the latter method.

If targets and effectors are mixed the higher cytotoxicity is possibly a reflection of a better contact between the effectors and the relevant target cell antigens. When the target cells are fixed to the bottom, it is reasonable to assume that a smaller proportion of target cell antigens is available for the CTLs to cause a lytic event. In our experience the higher cytotoxicity is reproducible and since this modification simplifies the procedure of the fluorochromasia method we have now adopted the mixing as standard technique.

For the final preparing of the trays for measurement there are two options, the removal of the released CF by replacement of the medium or the addition of a substance quenching the medium fluorescence without too much loss of cellular fluorescence. The latter approach is possible provided the quenching is achieved with a high molecular weight additive which does not enter the living cell and the sedimented cells are measured with an epi illumina-

Table 4
Comparison of CF CML assays with target cells fixed to the well bottom or mixed with the effectors

Effectors/targets		P[a]			S			G		
	E:T ratio	50:1	25:1	12:1	50:1	25:1	12:1	50:1	25:1	12:1
PS[c]		2[b]	2	4	24	20	7	7	1	6
PS[d]		4	6	9	46	48	44	22	13	15
PG[c]		2	5	1	7	5	5	41	33	29
PG[d]		3	0	3	27	27	16	54	52	46
GP[c]		44	29	23	13	5	6	1	14	3
GP[d]		44	42	31	26	20	11	2	7	3

[a] P: HLA-A1,25,B8,4?; Cw; DR1,3
S: A1,11; B7; Cw; DR
G: A1,21; B5[1],15; Cw3; DR1,2
[b] specific release
[c] Targets fixed to the well bottom
[d] Targets and effectors mixed

Table 5
Comparison of medium replacement and fluorescence quenching with hemoglobin

Effector[a]	target	I	M	C1	C2	C3	C4
C1 vs C2[b]		5	22	4	25	30	23
C1 vs C2[c]		3	27	4	31	29	25
C1 vs C4[b]		1	27	4	35	41	32
C1 vs C4[c]		11	31	9	36	32	36
C2 vs M[b]		5	38	43	3	6	3
C2 vs M[c]		2	27	31	−4	−2	4
C4 vs M[b]		10	59	57	2	16	1
C4 vs M[c]		29	61	64	6	16	9

[a] Effectors were made between members of Family 1 (see Table 1) and tested at 25 1 effector to target ratio

[b] Percentage specific release obtained when hemoglobin was added after the incubation to quench the medium fluorescence

[c] Percentage specific release obtained when after the incubation the released Cr was removed by rinsing with PBS

tion, inverted microscope As shown in Table 5 the addition of hemoglobin to quench the medium fluorescence led to results identical to those obtained by rinsing of the trays with medium The advantage of the addition of hemoglobin is that differences in fluorescent light output due to variable quenching by contamination with erythrocytes of the effector cells disappear by the very quenching of background fluorescence The addition of hemoglobin also avoids the possible loss of cells seen occasionally after the final rinse Although our experience is limited we feel the hemoglobin quenching method is a good alternative for the washing procedure used so far

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