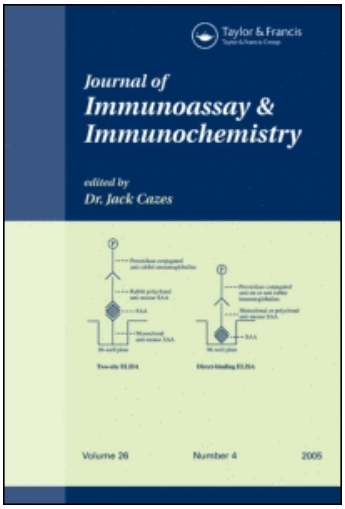

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A New Sensitive and Rapid Automated Fluorometric Assay for Detection of Natural Killer Activity Using Carboxyfluorescein Diacetate

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A NEW SENSITIVE AND RAPID AUTOMATED FLUOROMETRIC ASSAY FOR DETECTION OF NATURAL KILLER ACTIVITY USING CARBOXYFLUORESCEIN DIACETATE

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ABSTRACT

An automated fluorometric assay using carboxyfluorescein diacetate (CFDA) has been applied for the sensitive and rapid detection of natural killer (NK) activity. The lysis of target cells by NK cells was quantified by measuring the amount of CFDA released into the supernatant of culture wells with the aid of an automated microfluorometer. Both sensitivity and specificity of the presented method were higher than the ^{51}Cr release assay. Moreover, the detection of human NK activity against K562 target cells required only 2 hrs, compared to 4 hrs in the standard ^{51}Cr release assay.

'KEY WORDS: Fluorometry, NK assay, Carboxyfluorescein diacetate (CFDA)'

INTRODUCTION

The ^{51}Cr release assay has been used as the standard method for detection of natural killer (NK) activity (1). Recently, several fluorometric assays

have been applied to cell-mediated cytotoxic assay to avoid the use of radioactivity. Bruning et al. (2) reported that target cells, labeled with carboxyfluorescein diacetate (CFDA), were cultured with effector cells and that, at the end of the lysis, the fluorescence remaining in the cells after washing out released CFDA was read in an automated microfluorometer. McGuines et al. monitored the lysis of CFDA-labeled target cells by NK activity with laser flow cytometry (3). Similarly, Brenan and Parish developed a fluorometric assay for detecting cytotoxic T cells using Hoechst dye no. 33342 (4). In their system, however, there were still some difficulties in detecting the cell-mediated cytotoxicity with ease or short term. In the present study, we developed a sensitive and rapid automated fluorometric assay for detection of NK activity using CFDA.

MATERIALS AND METHODS

Animals

C3H/HeN ($H\text{-}2^k$) female mice, 6-10 week-old, were used.

Target cells

YAC-1 and RL♆-1 cells in exponential growth phase were used for mouse NK assay, and K562 were used for

human NK assay as targets. These cells were maintained in RPMI 1640 culture medium containing 10% fetal calf serum and antibiotics.

Effector cells

The spleen of C3H/HeN mice was minced to make a single-cell suspension, which was then passed through the nylon wool column (5). Enriched NK cells were used as effector cells in mouse NK assays. Heparinized peripheral blood was collected from healthy donors, and peripheral blood mononuclear cells were isolated by the lymphocyte separation medium. These mononuclear cells were resuspended in RPMI 1640 medium for use in human NK assay.

NK assay

A solution of CFDA (Wako Chemicals, Osaka, Japan) was prepared by diluting the stock solution (10 mg/ml stored in acetone at -80°C) in RPMI 1640 medium to 100 μg/ml and used within 15 min to avoid flocculation. Target cells were labeled with CFDA by resuspending cells in 5 ml of the working solution and incubating for 60 min at 37°C. In the ^{51}Cr release assay, the target cells were labeled with 100 μCi of ^{51}Cr by incubation for 60 min at 37°C (1). Labeled cells were washed 3 times and resuspended in RPMI 1640 medium.

CFDA or ^{51}Cr-labeled target cells and effector cells were suspended in 200 μl of culture medium per well of a U-bottom microplate. After centrifuging at 800 rpm for 1 min, the plates were incubated at 37°C in a humidified 5% CO_2 incubator. The plates were centrifuged after various incubation times as described above. In the CFDA release assay, 100 μl of the supernatant from each well was transferred into the corresponding well of another U-bottom microplate, and the fluorescence intensity in each well was measured with a microplate reader (MTP-32, Corona Electric Co., Ltd., Japan) fitted with 490 nm excitation and 530 nm emission filters. The machine was blanked on wells with medium only. For the ^{51}Cr release assay, 100 μl of the supernatant was harvested from each well and their radioactivity was assessed with a gamma scintillation counter. Spontaneous release was determined by incubating labeled targets alone, and maximal release was determined by addition of 0.5% NP-40. The following formula was used to estimate percent specific cytolysis:

$$\% \text{ Specific lysis} = \frac{\text{Experimental release (R)} - \text{spontaneous R}}{\text{Maximal R} - \text{spontaneous R}} \times 100$$

All samples were assayed in triplicate, and the values were presented as the mean ± one standard deviation of triplicate determinations.

TABLE 1.

Spontaneous release of CFDA from labeled YAC-1 target cells

Incubation time (hr)	% Spontaneous release			
	30 min labeling		60 min labeling	
	50 μg/ml	100 μg/ml	50 μg/ml	100 μg/ml
1	30 ± 1	17 ± 1	27 ± 1	15 ± 1
2	57 ± 2	32 ± 1	44 ± 1	34 ± 1
4	87 ± 1	75 ± 2	77 ± 2	73 ± 2

YAC-1 cells were labeled with 50 or 100 μg of CFDA for 30 or 60 min. Twenty thousand of labeled YAC-1 cells were cultured. The experiment represents one of three experiments.

Statistics. All statistical values presented were obtained using the Mann-Whitney U test.

RESULTS

Labeling of target cells

First, we tried to establish conditions suitable for labeling target cells with CFDA. The results are shown in Table 1. Spontaneous CFDA release from YAC-1 cells labeled at a concentration of 100 μg/ml was lower than that from cells labeled at a concentration of 50 μg/ml ($P<0.005$). In contrast, there was no marked

TABLE 2.

Comparison of spontaneous release between CFDA-labeling and ^{51}Cr-labeling

Incubation time (hr)	% Spontaneous release	
	CFDA	^{51}Cr
1	13 ± 1	2 ± 1
2	29 ± 1	3 ± 1
3	44 ± 2	5 ± 1

YAC-1 cells were labeled with CFDA (100 μg/ml) or ^{51}Cr (200 μCi/ml) for 60 min. Twenty thousand of YAC-1 cells were cultured. The experiment represents one of three experiments.

difference in spontaneous CFDA release from cells labeled for 30 or 60 min. Therefore, in the following experiments target cells were labeled with CFDA at a concentration of 100 μg/ml for 60 min. Next, we compared the rate of spontaneous release in labeling of target cells with CFDA or ^{51}Cr (Table 2). The spontaneous leakage of CFDA from target cells was higher than that in the case of ^{51}Cr at any time during incubation ($P<0.001$).

NK assay

The time course of NK activity using various numbers of CFDA-labeled YAC-1 target cells was followed

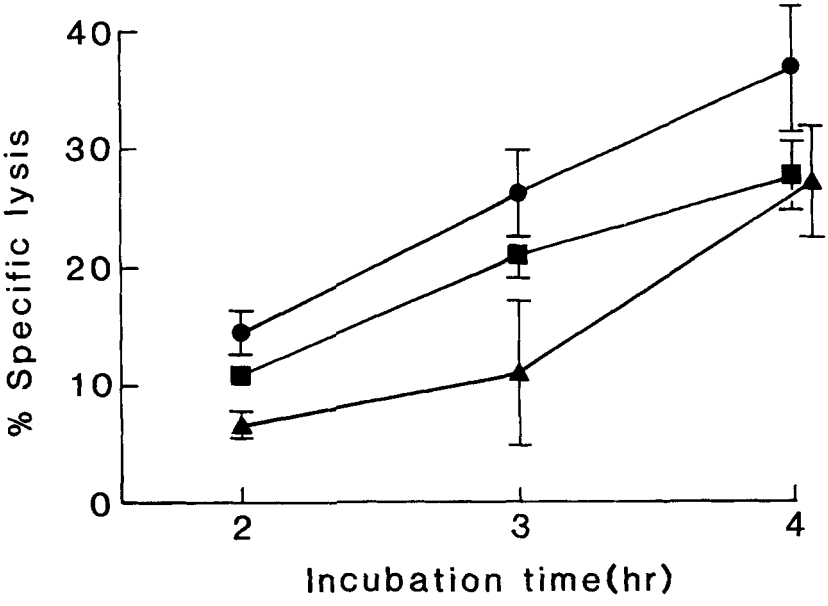

FIGURE 1. Time course of NK activity under the presence of various numbers of YAC-1 target cells: 2 (●), 1 (■), and 0.5 (▲) x 10^4/well of target cells were cultured. A E/T ratio was 40 :1. The experiment represents one of three experiments.

(Fig. 1). The specific lysis gradually increased during the incubation, and 3 hrs later a significant elevation was observed ($P<0.005$). At that time, an NK assay on 2 x 10^4 target cells per well was more efficient than that on 0.5 or 1 x 10^4 cells per well. At least, more than 1 x 10^4 target cells were required for the detection of NK activity. NK activity roughly paralleled the increase of the E/T ratio (Fig. 2). The

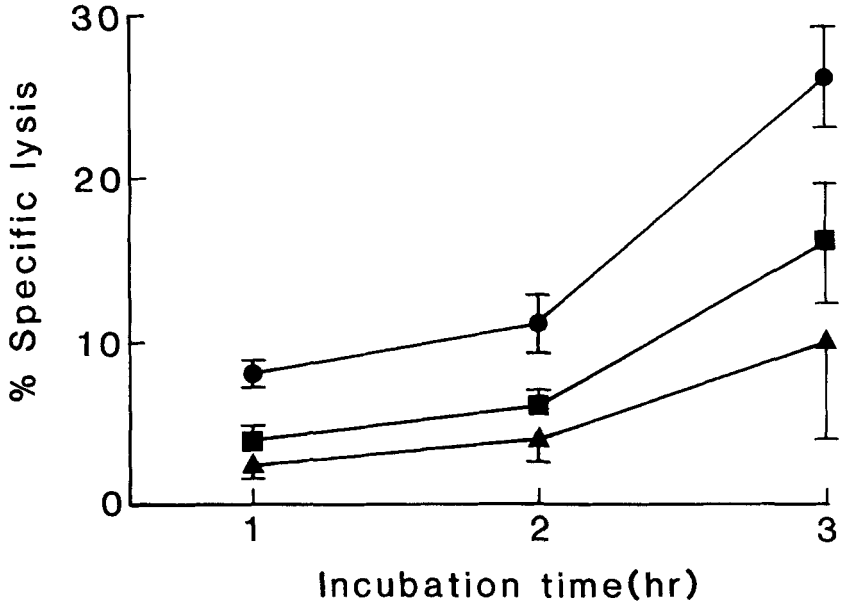

FIGURE 2. Time course of NK activity to RL♂-1 cells under the presence of various E/T ratios: 40 : 1 (●), 20 : 1 (■), 8 : 1 (▲). Twenty thousand cells of RL♂-1 cells were used. The experiment represents one of two experiments.

specific release of CFDA in this method was more than twice that of the ^{51}Cr release assay at two different E/T ratios (Fig. 3). The time course of NK activity in CFDA and ^{51}Cr release methods was compared (Fig. 4). The specific release of CFDA release method was more than twice that of the ^{51}Cr release assay during any incubation time. In the human NK assay system, we could detect definite NK activity even 2 hr after incubation,

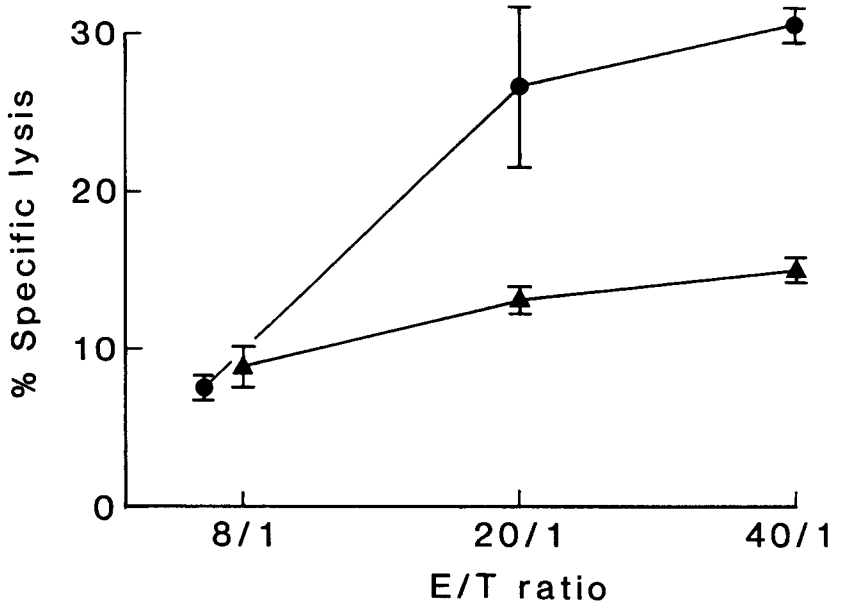

FIGURE 3. Comparison of NK activity of CFDA (●) and ^{51}Cr (▲) release method. YAC-1 target cells were cultured with effector cells for 3 hr. The experiment represents one of three experiments.

although it took 4 hr for the standard ^{51}Cr release assay. Further, NK activity was detectable at a low E/T ratio, such as 2:1 and 5:1 (Table 3). In addition, using NK cells, precultured with human recombinant IL-2 (20 U/ml) for 10 days, in this method induced extremely high specific release (about 100%) at a low E/T ratio (data not shown).

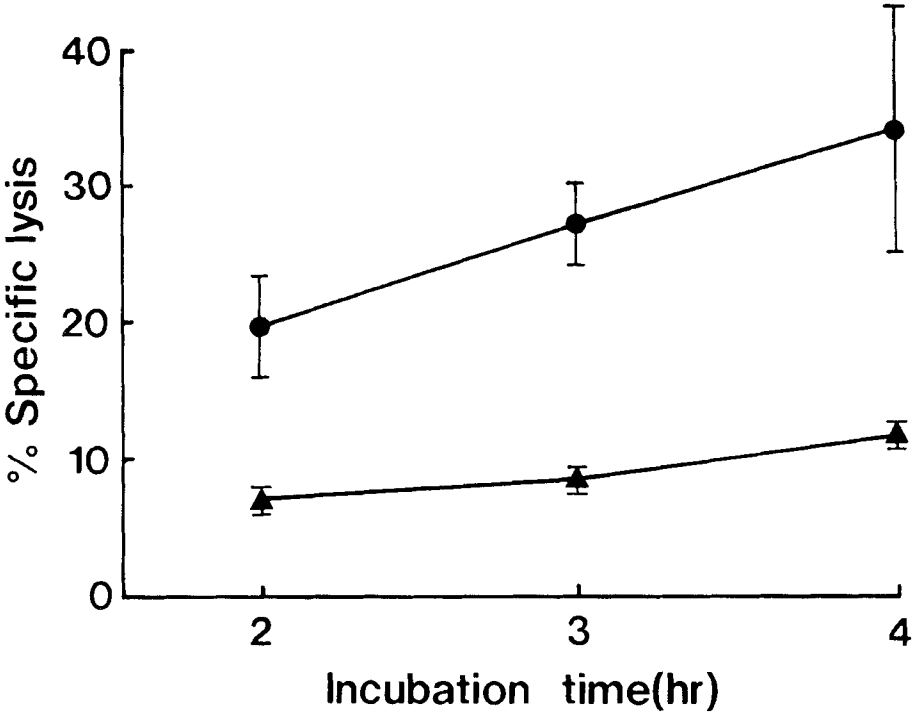

FIGURE 4. Comparison of the time course of NK activity between CFDA (●) and ^{51}Cr (▲) release method. YAC-1 target cells were cultured with effector cells at 40 : 1 E/T ratio. The experiment represents one of two experiments.

TABLE 3.

Fluorometric assay for human NK activity against K562 cells

E/T ratio	% Specific lysis	
	Exp. 1.	Exp. 2.
1	8 ± 1	10 ± 2
2	17 ± 2	22 ± 3
5	45 ± 5	39 ± 2
10	73 ± 2	66 ± 4

Twenty thousand of K562 target cells were cultured with effector cells for 2 hr. The experiment represents one of three experiments.

DISCUSSION

This paper describes a sensitive and rapid automated fluorometric method for detecting NK activity. This method is based on measuring the quantity of fluorescent CFDA released from CFDA-labeled target cells by cytotoxicity of NK cells. Our method using an automated microfluorometer (a microplate reader for the enzyme-linked immunoabsorbent assay) with appropriate filters can detect NK activity with rapidity and high sensitivity with no use of radioactivity, as compared with the ^{51}Cr release assay. In fact, our method requires only several minutes per plate for the assay. Since the fluorescence released into the culture supernatant is measured, the results would be essentially comparable to those obtained in ^{51}Cr release assay which also measures the released radioactivity.

The heavier labeling with CFDA (100 μg/ml vs 50 μg/ml) led to the lower % spontaneous release. The heavier load certainly increases the maximum release, whereas it does not much affect the spontaneous release. Therefore, the % spontaneous release in the heavy labeling becomes relatively lower. The spontaneous release of CFDA from labeling cells at the

long incubation time was higher than that of ^{51}Cr labeling. This may be closely associated with the high sensitivity of the CFDA release assay. Possibly, this method could be applied to the other short term cytotoxic assay, such as complement-dependent cytotoxic assay. However, it may not be suitable to perform the T cell cytotoxic assay requiring overnight incubation.

There are several reports on the cytotoxic assay using CFDA. It is necessary to avoid mixing effector cells and target cells in the method reported by Bruning et al. (2), because their method measures the fluorescence remaining in labeled target cells. Therefore, the method may not detect an accurate NK activity because of the insufficient contact between target cells and effector cells. The CFDA assay using the laser flow cytometer (3) also measures the fluorescence intensity remaining in labeled cells. This method take a relatively long time for the assay of each sample, and needs the laser flow cytometer. These methods are different from the release assay, such as our method and the ^{51}Cr release assay. Another fluorometric assay using Hoechst dye no.33342 (4) are insensitive for short term assay, such as 4 hr. Our method might be operated more easily, stably and sensitively except for requiring an automated fluorometer.

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