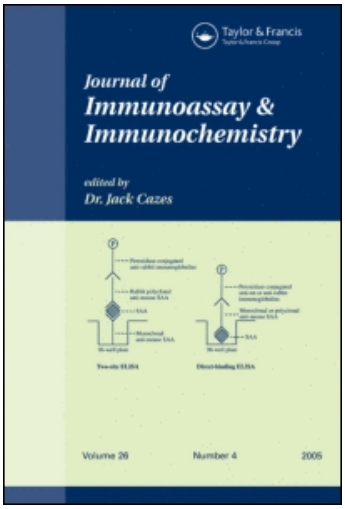

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Development of Enzyme Immunoassay for Platonin® (NK 19)

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DEVELOPMENT OF ENZYME IMMUNOASSAY FOR PLATONIN® (NK 19)

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ABSTRACT

A sensitive and specific enzyme immunoassay for an immunomodulator PLATONIN (NK 19) has been developed and applied to the evaluation of pharmacokinetics in human and rats. NK 19 analog, succinyl NK 19 has been synthesized and coupled to bovine serum albumin. This conjugate is immunogenic and when injected into rabbits antibodies of high affinity for NK 19 were obtained. Some light decomposed products of Platonin were of lower cross-reactivity with these antibodies. For competitive reactions, these antibodies were incubated with a mixture of succinyl NK 2900-β-D-galactosidase conjugate and standard NK 19 or sample. Then, antibody-bound enzyme-hapten were separated from free one using anti-rabbit IgG immobilized on polystyrene balls. Activity of the enzyme on the solid phase was fluorometrically determined. The present immunoassay allows detection of as low as 10 pg of NK 19 in the biological fluids using 50 μl of the samples. The accuracy and reproducibility were also quite satisfactory. By use of this assay, plasma levels of NK 19 after a single oral administration of 5 mg or 10 mg/body to human volunteers or intravenous injection of 2 μg/kg to rats could be determined.

KEY WORDS: enzyme immunoassay, Platonin®, NK 19, immunomodulator, β-D-galactosidase, plasma levels of NK 19

 0197-1522/86/0701-0017$3.50/0

Abbreviations used in this paper: EIA; enzyme immunoassay, β-Gal; β-D-galactosidase, BSA; bovine serum albumin, NK 19; PLATONIN®, EDC; 1-ethyl-3 (3-diethylaminopropyl) carbodiimide hydrochloride

INTRODUCTION

Platonin®(NK 19) is a photosensitive cyanin dye that has been used for the treatment of wounds and burns (1-4). Recently, Yamamoto et al. have demonstrated that this dye is a typical immunomodulator capable of modifying immunological function of the impaired lymphocytes (5-9). Thereafter, clinical studies of this compound for rheumatoid arthritis have been accumulated (10-15).

For more detailed studies of pharmacological actions and pharmacokinetics of NK 19, the availability of sensitive and specific assay methods is of great importance. Spectrophotometric assay method has been used for quantitative determinations of NK 19 (16). A drawback of this method is, however, the low sensitivity and is not suitable for determination of plasma levels of NK 19 after clinical doses of oral administrations.

This report describes development of an enzyme immunoassay for NK 19 which is superior to the previous spectrophotometric method both with regard to sensitivity and specificity.

MATERIALS AND METHODS

Reagents

NK-19 (Platonin) (2,2'-[3-[2-(3-heptyl-4-methyl-4-thiazolin-2-ylidene) ethylidene] propenylene] bis [3-heptyl-4-methylthiazolium iodide]) and NK 2900 (2,2'-[3-[2-(3-(2-hydroxyethyl)-4-methyl-4-thiazolin-2-ylidene) ethylidene] propenylene] bis [3-heptyl-4-methylthiazolium iodide]) and NK 19 decomposed products, 3-heptyl-4-methylthiazolium iodide (HMT) and 2,4-dimethyl-3-heptylthiazolium iodide (DHT) were donated by Nippon Kankoshikiso Kenkyusho, Okayama. Chemical structures of them are illustrated in Figure 1. β-D-Galactosidase (β-Gal, from Escherichia coli, Grade VIII, 940 units/mg protein), and bovine serum albumin (BSA, Cohn fraction V, lyophilized) and 4-methylumbelliferyl-β-D-galactoside (4-MUG) were purchased from Sigma Chemical Co. (St, Louis, Mo.); Immunoglobulin G (IgG) fraction of anti-rabbit IgG (prepared in goat, lyophilized), from Miles-Yeda Ltd. (Islael); Freund's complete and incomplete adjuvants (FCA and FIC), from Difco Lab. (Detroit, Mich); Sephadex LH 20, from Pharmacia Fine Chemicals (Upssala, Sweeden); 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) from E. Merck AG (Darmstadt); Polystyrene balls (diameter, 1/4 inch), from Ichiko Co., Ltd. (Nagoya); succinic anhydride, from Ishizu Pharmaceutical Co. Ltd. All other chemicals from commercial sources were of analytical grade quality.

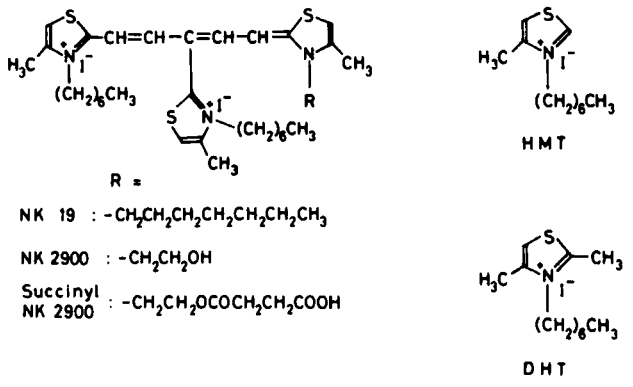

FIGURE 1 Chemical structure of Platonin (NK 19) and its related compounds.

Buffers used for the assay

A_1 buffer: 0.01 M sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl,1 mM $MgCl_2$ and 0.1 % BSA.

A_2 buffer: 0.1 M sodium phosphate buffer (pH 6.7) containing 0.1 M NaCl, 1 mM $MgCl_2$ and 2 % BSA.

Animals

Male albino rabbits weighing approximately 2 kg were used for the immunization (Yanahara rabbit center, Tsuyama). Male Wister rats weighing 400 g were used for pharmakodynamic study (Charles-River Japan Co. Ltd., Atsugi, Kanagawa).

Volunteers

Seven healthy male adults were subjected to this study.

Administration of NK 19 and collections of blood

The cupsules each containing 50 -200 μg NK 19 were orally administered after an overnight fast with 100 ml of water. Blood samples were taken from the antecubita vein into vacuum blood collection tubes treated with heparin before and 0.5, 1, 2, 3 and 4 hrs after administration. After the blood collection, plasma were prepared by an usual method and stored at -20°C before measurement.

Preparation of immunogen

NK 19 has not any appropriate chemical groups which can conjugate with a carrier protein in the molecule. Thus, NK 19 analog, NK 2900 was synthesized and succinylated, and was conjugated to BSA. Briefly, 2 mg NK 2900 was dissolved in 1 ml of anhydrous pyridine followed by the addition of 150 mg succinic anhydride, and incubated for 4 hrs at room temperature under the dark condition. Reaction was stopped by the addition of 100 μl of distilled water. The mixture was evaporated and the reactant was disolved in 500 μl of methanol. Succinyl NK 2900 was purified by Sephadex LH 20 column chromatography and the solvent was evaporated. The succinyl NK 2900 was disolved in 500 μl of 0.1 M phosphate buffer (pH 6.5) followed by the addition of 10 mg EDC in a total volume of 1 ml. Ten mg of BSA was then added dropwise with constant stirring. The mixture was allowed to react for 24 hr at 4°C with stirring under the dark condition.

The resultant conjugate was purified by dialysis and then lyophilized. Thus obtained conjugate was stored at -20°C. Protein assay value by Lowry-Follin method and spectrophotometric determination of hapten indicated a conjugate of about 7 succinyl NK 2900 residues per BSA molecule.

Immunization

Antibodies to NK 19 was produced in rabbits by repeating intradermal injections of the immunogen. The first immunization was performed with 0.5 mg of succinyl NK 2900-BSA conjugate emulsified in Freund's complete adjuvant and the booster doses of 0.25 mg of the immunogen emulsified in Freund's incomplete adjuvant were given at two weeks intervals. The titre of the antibody was frequently inspected employing enzyme immunoassay (EIA) and bilayer precipitin method as described by Yamamoto et al (17-19). On the 7th day after the last booster, blood was taken from the carotied artery. The antisera obtained were lyophilized and stored at -20°C until use.

Preparation of hapten-enzyme conjugate

The procedure described below were performed at 4°C under the dark condition. Succinyl NK 2900 was conjugated to β-Gal with EDC. Briefly, One mg of succinyl NK 2900 was disolved in 400 µl of 0.1 M phosphate buffer, pH 6.5 followed by the addition of 40 µl (10 mg/ml) of EDC. One hundred µl of β-Gal (1

mg protein/ml) was then added dropwise with constant stirring. After standing for 4 hrs, the preparation was subjected to dialysis against the same buffer. The enzyme activity was kept, as same as the original source. The hapten-enzyme conjugate thus obtained was kept at -20°C after mixing with equivalent volume of glycerin.

Preparation of second antibody-bound polystyrene balls

Second antibody was immobilized on polystyrene balls by physical adsorption according to the manner described in our previous paper (19). Briefly, balls were soaked in a solution of the goat IgG fraction of anti-rabbit IgG (20 mg) in 100 ml of 0.05 M phosphate buffered saline, pH 7.5 containing 0.1 % NaN_3 at 4°C for 24 hrs and washed three times with A_1 buffer. They were kept in the same buffer at 4°C for 18 - 24 hrs. The buffer, then, was changed with new A_1 buffer containing 1 % BSA and kept another one hour until used to prevent antificial interactions of samples with the coated balls.

Assay procedure

The enzyme immunoassay for NK 19 involved competition of β-Gal labeled succinyl NK 2900 and standard or sample for a limitted amount of antibody with separation of free and antibody-bound by anti-IgG immobilized on polystyrene balls. Finally, enzyme activity on the solid phase was determined as described in

our previous papers (17-19). Briefly, fifty µl of standard or sample solution was incubated at 4°C for 18 hrs with 50 µl of the enzyme labeled hapten (diluted 1 : 20,000 with A_2 buffer) and 50 µl of anti NK 19 serum (diluted 1 : 30,000 with A_2 buffer). Then, a second antibody immobilized polystyrene ball was added to each assay tube. After mixing for 4 hrs at room temperature, the balls were washed with A_1 buffer and transfered to a tube holding 200 µl of A_1 buffer and 200 µl of 0.3 mM 4 MUG. After incubation for 2 hrs at 37°C, 1.6 ml of 0.1 M Na_2CO_3 solution was added to stop the reaction. The amount of 4-MU liberated was determined by fluorescence spectrophotometry with an excitation wavelength at 360 nm and emission wavelength at 450 nm.

B/B_0 (%) was calculated as following equation.

$$B/B_0 = \frac{B - BL}{B_0 - BL} \times 100$$

Where,

B : fluorescent intensity of sample or standard

B_0: fluorescent intensity of control(lack of unlabelled hapten)

BL: fluorescent intensity of blank (lack of antibody)

RESULTS

Calibration curve and reactivity of enzyme immunoassay for NK 19

A typical calibration curve for the enzyme immunoassay of NK 19 shows a displacement of enzyme labeled succinyl NK 2900 by

unlabeled NK 19, when plotted as a semilogarithmic function from 10 to 500 pg per tube of NK 19 (Fig. 2). This figure also shows the displacement curve for NK 2900 and succinyl NK 2900 which are the immunogenic haptens synthesized and used for raising antibody to NK 19, because NK 19 lacks of appropriate chemical group to conjugate with carrier protein on its molecule. As expected, sensitivities for NK 2900 and succinyl NK 2900 were shown to be much higher than that of NK 19 on this enzyme immunoassay. Concentrations of NK 19, NK 2900 and succinyl NK 2900 showing meadian (50 %) inhibitory activities were proved to be 70, 30 and 10 pg/tube, respectively. Examination of their direct inhibition on the β-Gal activity revealed that all of these haptens have no effect on this enzyme activity. Fluorescence intensity was not also influenced by these haptens (data not shown). The specificity of antibody directed against NK 19 was assessed with some of known light-decomposed products of NK 19, namely HMT and DHT. The results showed no practical cross-reactivity of these compounds on this assay system (Fig. 2).

Reliability of the assay system

Recovery and dilution test were carried out to examine inhibition of enzyme or of Ag-Ab reaction due to non-specific interfering substances in biological fluids or to rabbit IgG. Dilution tests were performed with plasma samples which were serially diluted 1 : 2 to 1 : 32 with A_2 buffer. This experiment

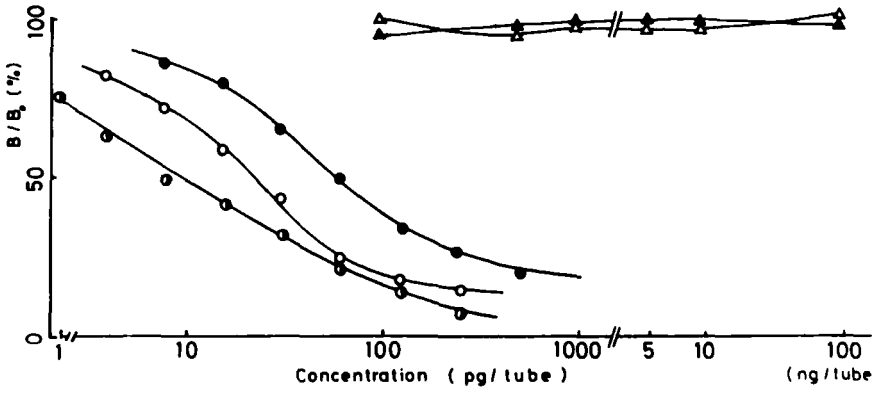

FIGURE 2 Calibration curves for Platonin (NK 19) and its related compounds. Each point represents the mean of triplicate determinations according to the assay procedure. (●); Platonin (NK 19), (○); NK 2900, (◑); Succinyl NK 2900, (▲); HMT, (△); DTH.

resulted in a good linear relationship between dilution and measured values (Table I). Recovery studies with mean recoveries of 97 % (low concentration of NK 19 added) and 119 % (high concentration of NK 19 added) are shown in table II.

Plasma levels of NK 19 in healthy volunteers after a single dose of oral administration

The proposed assay was applied to the determination of NK 19 in plasma samples from 7 men who were treated orally with a single dose of 5 mg or 10 mg of NK 19 capsules (one capsule containing 500 μg). Fig. 3 shows the time course of plasma concentrations of NK 19. The levels were observed to rapidly increase reaching at maximum within one hour after the oral

TABLE 1

Dilution Test of NK-19 in the Proposed Enzyme Immunoassay

Dilution	Measured value (pg/tube)	Concentration of original sample (ng/ml)
1 : 32	6.8	4.35
1 : 16	15	4.80
1 : 8	27	4.32
1 : 4	58	4.64
1 : 2	100	4.00
Mean ± S.D.		4.42 ± 0.31

The concentrations of Platonin (NK 19) were caliculated using the calibration curve. Samples were prepared by stepwise dilutions of original Platonin solution with normal plasma.

administration, except one subject in the case of 5 mg administration, although only one point of the subject was found to be aberrant at 3 hrs by unknown reasons, but not by the reproducibility of the assay. The levels were rapidly decreased and were lower than 1/10 of the maximum concentration in 4 hrs after the administration. The half life of NK 19 in blood was estimated to be 2.5 hrs (A):5mg dose, B):10 mg dose).

TABLE 2

Recovery Test of NK-19 in the Proposed Enzyme Immunoassay

Sample	Added NK-19 (pg/tube)	Measured value (pg/tube)	Recovery (%)	Mean ± S.D.
1		21	105	
2		19	95	
3	20	22	110	97 ± 10
4		17	85	
5		18	90	
6		110	110	
7		120	120	
8	100	115	115	119 ± 8
9		130	130	
10		120	120	

The concentrations of Platonin (NK 19) were caliculated using the calibration curve. Samples were prepared by adding known amounts of Platonin to Platonin free plasma.

Plasma levels of NK 19 in healthy volunteers after a single dose of intravenous injection

The plasma levels were observed after intravenous injection of 100 μg of NK 19 to 2 volunteers. The maximum concentration reached at 15 min after the injection, and then rapidly decreased

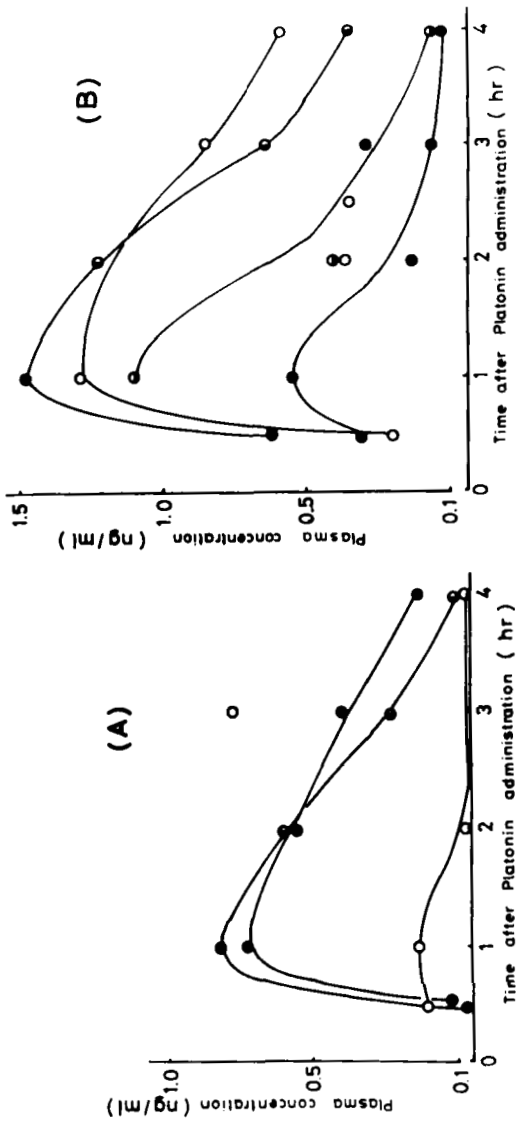

FIGURE 3 Plasma levels of Platonin (NK 19) in healthy volunteers after a single dose of oral administration. Each point represents the mean of triplicate determinations of each volunteer. Panel A; 5 mg dose (Platonin 1 mg capsule x 5) p.o., Panel B; 10 mg dose (Platonin 1 mg capsule x 10) p.o..

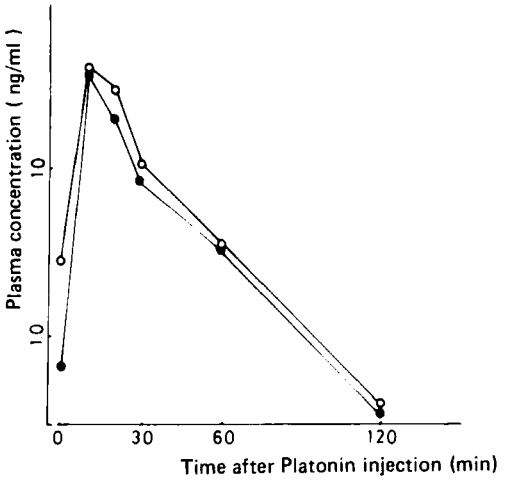

FIGURE 4 Plasma levels of Platonin (NK 19) in healthy volunteers after a single dose of intravenous injection. Each point represents the mean of triplicate determinations of each volunteer. Dose; 100 μg Platonin capsule to each volunteer.

(Fig. 4). In 120 min after the injection, only a trace of NK 19 has been detected in plasma.

Plasma levels of NK 19 in rats after a single dose of intravenous injection

Two male rats (about 400 g) were treated intravenously with NK 19 at a dose of 2 μg/kg. It was also observed that the plasma levels of NK 19 rapidly disappeared (Fig. 5). The half life of NK 19 was estimated to be 15 min after the injection.

DISCUSSION

Recently, immunomodulating activity of Platonin (NK 19) has been demonstrated by Yamamoto et al. with animal models (5-9).

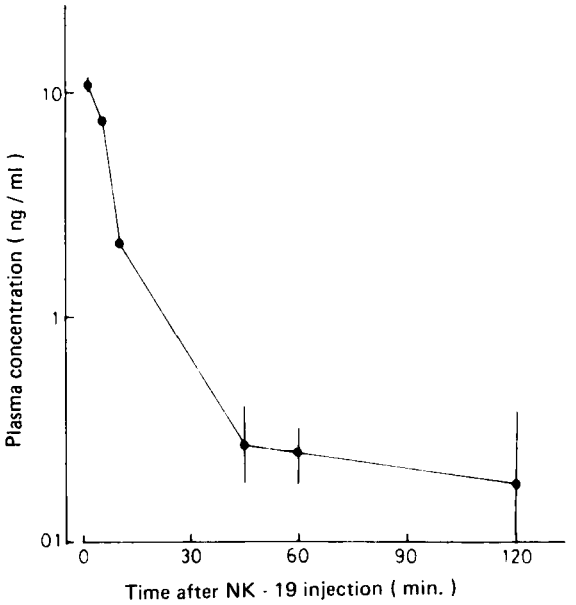

FIGURE 5 Plasma levels of Platonin (NK 19) in rats after a single dose of intravenous injection. Each point represents the mean ± S.E. of triplicate determinations from two male rats.

They showed that loading of restraint stress to mice resulted in suppression of anti-SRBC PFC response and that Platonin treatment significantly rescued the mice from the suppressed states in terms of the immune responses (5,7). Platonin treatment also inhibited the elevation of natural thymocytotoxic autoantibody (NTA) in spontaneously hypertensive rats (SHR) and NZB/NZW F_1 mice when they were treated at a dose of 1 μg/kg (p.o.) for more than 3 months (8,9). Oral administration of Platonin inhibited the adjuvant or type II collagen induced-arthritis in Lewis rats (5,7,8). The most effective dose of Platonin was 0.05 μg/body

/day. However, the carrageenan-induced inflammation was not inhibited by Platonin administration in rats even if any higher doses were given to them (7). These findings suggested that Platonin is a typical immunomodulator which may be effective to treatment of immune disorders, especially autoimmune diseases such as rheumatoid arthritis. Clinical evaluations of Platonin on rheumatoid arthritis suggest this drug is significantly effective to this disease as well as to wounds and burns (10-15). Only 50-100 μg/body doses of Platonin were used by treatments of rheumatic patients in these clinical studies (10,15). The mechanisms of action of this agent remain to be unsolved. In addition, investigation of pharmacokinetics of human plasma levels of Platonin is necessary to evaluate the effectiveness in more details. Sensitive and specific assay methods for Platonin are not available. We have established the enzyme immunoassay for Platonin in this paper.

Succinyl NK 2900 was synthesized and used as hapten in an attempt to produce antibody against NK 19, since NK 19 has no chemical group which is capable of conjugating the carrier protein, BSA, in its molecule. The antibody raised by injections of the immunogen, succinyl NK 2900 BSA conjugate displayed favourable affinity to NK 19, although this particular antibody possessed higher affinity to NK 2900 or succinyl NK 2900. NK 2900 and succinyl NK 2900 are not considered to be metabolites of NK 19. In addition, HMT and DHT which are light decomposed

products and also putative metabolite of NK 19 *in vivo* did not cross react in an amount up to 100 ng per tube. Thus, we have been able to establish a specific and sensitive enzyme immunoassay for NK 19 by use of this antibody. This assay made it possible to detect as low as 10 pg of NK 19 in the biological fluids using 50 μl of the sample without treatment procedure such as deproteinizing prior to the assay.

A spectrophotometric assay for NK 19 has been reported (16). However, this assay has not been applied for clinical studies, because of lack of specificity and sensitivity. Thus, this is the first report to describe the plasma levels of NK 19 in human after oral and intravenous administrations. This experiment has demonstrated that plasma levels of NK 19 after a single dose of NK 19 rapidly decreased. These findings suggest that effective concentration of NK 19 in blood is very low or that pharmacological activity of NK 19 is not dependent upon its blood levels. Mode of action of NK 19 is now in progress in our laboratory. A preriminary experiment exhibits that the assay method using NK 2900-β-Gal conjugate prepared with Br_2 and triphenylphosphine, is nearly twice as sensitive as the assay described here with succinyl NK 2900-β-Gal.

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