Immunology 1979 **36** 235

Rhodamine as a fluorescent probe of lymphocyte activation

R. C. NAIRN, IRENE M. JABLONKA, JENNIFER M. ROLLAND, G. M. HALLIDAY & H. A. WARD *Department of Pathology and Immunology, Monash University Medical School, Melbourne, Australia*

Received 4 *April* 1978; *accepted for publication* 18 *May* 1978

Summary. Fresh rat and mouse lymphoid cells have been labelled by stable linkage with tetramethylrhodamine isothiocyanate (TMRITC). A change in intensity, either an increase or a decrease of the fluorescent emission of the cells, detected by microfluorimetry, was induced by mitogen stimulation or the mixed lymphocyte reaction. The change in fluorescence was observed within 3 h of mitogen stimulation and within 0·5 h in the mixed lymphocyte test. These early cellular responses were detectable consistently whether the labelling was done before or after mitogen stimulation; post-labelling only was studied in the mixed lymphocyte reaction. The method should provide a time-saving practical procedure for early detection of the lymphoid cell responses and would readily lend itself to flow cytofluorimetry for possible routine diagnostic use.

INTRODUCTION

A simple fluorescent cell probe method for monitoring molecular changes in lymphocyte surface membranes would permit the detection and facilitate the study of changes within a few hours of cell activation. We have labelled fresh rat and mouse lymphoid cells with tetramethylrhodamine isothiocyanate (TMRITC). A response to mitogens was detectable using cells labelled either before or after mitogenic stimulation, and a response was also detected to the mixed lymphocyte reaction. The response was observed as a change in intensity, either an increase or a decrease, of the fluorescent emission of lymphoid cells, examined by incident light fluorescence microscopy and microfluorimetry.

Correspondence: Professor R. C. Nairn, Department of Pathology and Immunology, Monash University Medical School, Prahran, Victoria 3181, Australia.
0019-2805/79/0200-0235$02.00

MATERIALS AND METHODS

Lymphoid cell suspensions were made from fresh thymus, lymph nodes or spleen of adult DA Agouti rats, BALB/c mice and C3H mice. Immediately after removal from the animal, the lymphoid organs were teased with hypodermic needles in phosphate-buffered saline (PBS: 0·145 M NaCl, 0·01 M sodium phosphate, pH 7·1). The resulting cell suspension was washed three times in PBS by centrifugation at 250 g_{max} for 5 min periods and the cells were resuspended (10^7 cells/ml). T-cell enriched fractions of lymph nodes and spleen were obtained by removal

of EAC-rosetting cells from the original cell suspensions (Matthews, Chalmers, Flannery & Nairn, 1976).

For blood lymphocyte suspensions, heparinized blood (lithium heparin, 12·5 iu/ml blood) was diluted in an equal volume of PBS, layered on Hypaque-Ficoll (34% Hypaque (Winthrop), 9% Ficoll 400 (Pharmacia), 5:12) and centrifuged at 250 g_{max} for 25 min. The interface layer of cells was removed, washed three times in PBS and resuspended (10^7 cells/ml).

Lymphocyte stimulation

For studying mitogen effects, cells were incubated with an equal volume in PBS of 1:16 phytohaemagglutinin (PHA, Wellcome, reagent grade HA 15) or 1:400 pokeweed mitogen (PWM, Grand Island Biological; reconstituted as directed), or 0·1% concanavalin A (Con A, Sigma Chemical Co.) for 3 h at 37° in 5% CO_2 in air. Control preparations were diluted with an equal volume of PBS and incubated in the same way as the tests. At the end of the incubation all preparations were washed three times in PBS. Additional controls were provided by azide inhibition of the PHA stimulation: sodium azide was added at a final concentration of 5×10^{-3} M to test and control cell suspensions.

Mixed lymphocyte reactions were assessed by mixing equal numbers of non-immune syngeneic BALB/c and C3H mouse thymocytes in Hanks's balanced salt solution (pH 7·2) at 37° for 0·5, 1, 3 or 7 h. In a control experiment for comparison, sodium azide was used to prevent stimulation, at the same 5×10^{-3} M concentration.

Rhodamine labelling of lymphocytes

This was carried out either before or after mitogen stimulation, and after mixed lymphocyte stimulation. The cells were suspended in 0·5 ml of labelling buffer (0·069 M NaCl, 0·007 M KCl, 0·05 M Na_2HPO_4, pH 8·6). For conjugation, 5μl of 0·1 mg/ml TMRITC (Crystalline isomer R, BBL, BioQuest) in the pH 8·6 labelling buffer were added to the cell suspension which was agitated gently for 3 min at room temperature. The total volume was then made up to 5 ml with PBS and the suspension centrifuged for 5 min at 250 g_{max}. The supernatant was removed, and the cells were resuspended in 1 ml of PBS and dialysed overnight at 4° against PBS containing free washed activated powdered charcoal (British Drug Houses) (Nairn, 1976). Finally, the cells, centrifuged at 250 g_{max} for 5 min, were resuspended in a few drops of PBS/glycerol, 1:1 mixture, for microscopic examination. Stable labelling of the cells was confirmed by microscopy: there was no detectable leaching of fluorochrome into the mounting medium during the period of examination. Indeed in a preparation kept for the particular purpose of long term assessment of labelling stability, the cells remained brightly fluorescent and there was no leaching of fluorochrome into the mountant even after 18 months storage at 4°. The chemical nature of the fluorochrome linkage to the cells has not been investigated but the labelling conditions including pH were chosen to give maximum opportunity for covalent bonding to cell proteins while preserving the viability of the cells.

Fluorescence microscopy and microfluorimetry

The lymphoid cells in 1:1 PBS/glycerol were mounted on ordinary glass microscope slides under 22 mm square coverslips and sealed with finger-nail varnish. A Leitz Orthoplan fluorescence microscope fitted with a Ploemopak II incident illuminator was used to illuminate the rhodamine-labelled cells by narrow-band green light (510–565 nm) near the absorption maxima of TMRITC conjugates (518 and 554 nm) (Nairn, 1976). The Leitz ×50 water immersion objective with a ×6·3 ocular gave the best image for the microfluorimetry.

The Leitz MPV2 microphotofluorimeter was used to measure, at the fluorescence emission peak of 615 nm, ×50 objective fields each selected to contain 4–10 cells. These were first located by tungsten light darkground microscopy to prevent bleaching of the specimen before fluorimetry, and the emission intensity of 10–20 such fields was assessed by the fluorimeter by switching from the transmitted tungsten light to the incident narrow-band green. Specimens were read 'blind' and in random order to avoid observer bias in the fluorimetry. Possible variations after mitogen stimulation in emission wavelength maximum over the range 580–650 nm were also monitored by adjusting the wavelength by the continuous wedge interference filter of the MPV2 in a number of experiments; no such variations were detected and the emission peak of the labelled cells remained at 615 nm. It is interesting that the emission peak of TMRITC-labelled γ-globulin is at a lower wavelength (573 nm).

Viability of cells after TMRITC labelling was checked in each experiment by the dye exclusion test

with 0·1% trypan blue which showed that over 90% of cells were viable.

Statistical evaluation

The Wilcoxon two-sample test (Sokol & Rohlf, 1969) was used.

RESULTS

Mitogen stimulation

The results of mitogen stimulation of different lymphoid cell populations from rat and mouse are summarized in Tables 1 and 2. Table 1 shows the effect of PHA, Con A and PWM on lymphoid cells from rat thymus and lymph nodes, and BALB/c and C3H mouse thymus. Cells were labelled with TMRITC after mitogen stimulation. In all experiments there was a significant difference in fluorescence of the stimulated cells compared with the controls: fluorescence emission was increased in PHA- and Con A-stimulated cells, and decreased in the PWM- stimulated cells.

Table 2 shows the effect of PHA on lymphoid cells already labelled with TMRITC. Thymus, blood, lymph node and spleen cells were studied. Experiments on spleen and lymph node and their putative T-cell enriched fractions (i.e. depleted of EAC-rosetting cells) are also recorded. In all cases, the fluorescent emission is significantly enhanced by the

Table 1. Mitogen stimulation at 37° for 3 h of rat and mouse lymphoid cells followed by TMRITC labelling

Cell Source	Stimulation	*Mean fluorescence per cell at 615nm in arbitrary units ± S.D. Control	Test	Significance (Wilcoxon test) *P*
Rat thymus	PHA	84·7 ± 6·0	106·0 ± 9·0	< 0·001
Rat thymus	PHA	71·5 ± 15·8	85·3 ± 23·0	< 0·01
Rat thymus	PHA	71·5 ± 15·8	91·4 ± 18·0	< 0·001
Rat thymus	Con A	19·9 ± 10·3	32·3 ± 13·5	< 0·001
Rat cervical lymph node	PHA	58·0 ± 19·7	84·8 ± 21·4	< 0·001
Rat cervical lymph node	PWM	33·3 ± 15·0	22·0 ± 9·5	< 0·005
BALB/c mouse thymus	PWM	103·9 ± 15·5	58·5 ± 25·4	< 0·001
C3 H mouse thymus	PWM	83·0 ± 24·1	53·3 ± 23·6	< 0·001

* 20 measurements per sample, except for the first rat thymus experiment, with 10 measurements.

Table 2. Populations and sub-populations of rat and mouse lymphoid cells labelled with TMRITC and then stimulated with PHA

Cell source	*Mean fluorescence per cell at 615 nm in arbitrary units ± S.D. Control	Test	Significance (Wilcoxon test) *P*
Rat thymus	13·8 ± 5·1	22·7 ± 7·8	< 0·001
Rat thymus	10·1 ± 2·8	17·8 ± 4·3	< 0·001
Rat blood	6·3 ± 1·7	9·2 ± 2·4	< 0·001
Rat cervical lymph node	9·9 ± 4·4	19·7 ± 11·5	< 0·001
Rat cervical lymph node	5·4 ± 1·8	7·4 ± 2·2	< 0·002
Rat cervical lymph node	21·8 ± 7·4	33·2 ± 9·9	< 0·001
Rat cervical lymph node	15·4 ± 3·4	23·6 ± 5·6	< 0·001
Non-EAC-rosetting cell fraction	17·0 ± 5·3	20·9 ± 4·8	< 0·01
Rat spleen	12·5 ± 2·5	21·2 ± 3·5	< 0·001
Non-EAC-rosetting cell fraction	8·2 ± 2·3	17·0 ± 2·3	< 0·001
C3 H Mouse thymus	73·6 ± 13·4	90·3 ± 29·9	< 0·001

* 15 measurements per sample, except for rat blood (105) and mouse thymus (100).

PHA stimulation, but no additional effect could be detected from the cell fractionation.

Figure 1 shows azide inhibition of PHA stimulation of TMRITC-labelled rat lymphoid cells. In the same experiment, cells not treated with azide gave the usual increase in fluorescence after PHA stimulation.

Mixed lymphocyte reaction

Four experiments were performed comparing fluorescence changes in the mixture of the BALB/c and C3H mouse thymocytes with levels in unmixed thymocytes from both strains. The emission from the mixed cells, measured after 0·5, 1, 3 and 7 h respectively, fell below the values for either strain alone in the 3 h experiment ($P < 0{\cdot}001$) (Fig. 2a), and between the values for either strain in the other three experiments, but significantly lower ($P < 0{\cdot}001$) than the mean position between the two individual cell types (e.g. Fig. 3). In a parallel 3 h mixed lymphocyte experiment lymphocytes pre-treated with sodium azide to prevent stimulation showed no significant

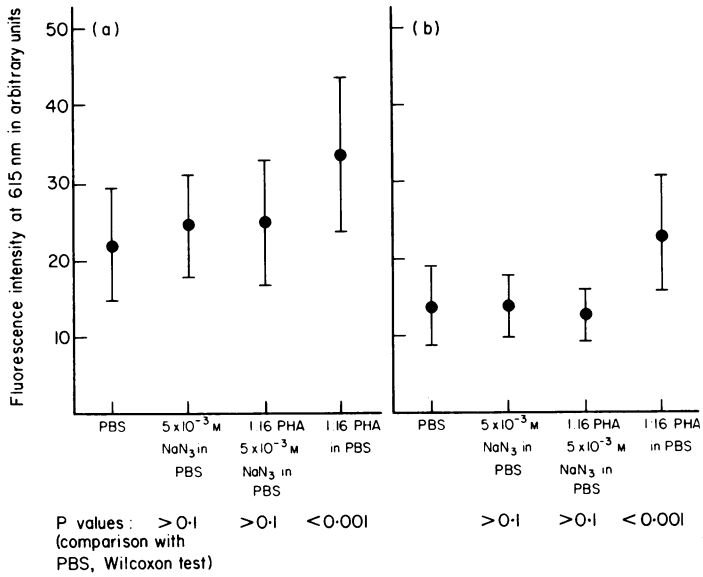

Figure 1. Effect of sodium azide on PHA-stimulated and unstimulated TMRITC-labelled cells (15 microscopic fields, containing approximately 100 total cells, counted in each experiment). (a) Rat lymph node lymphocytes; (b) Rat thymocytes.

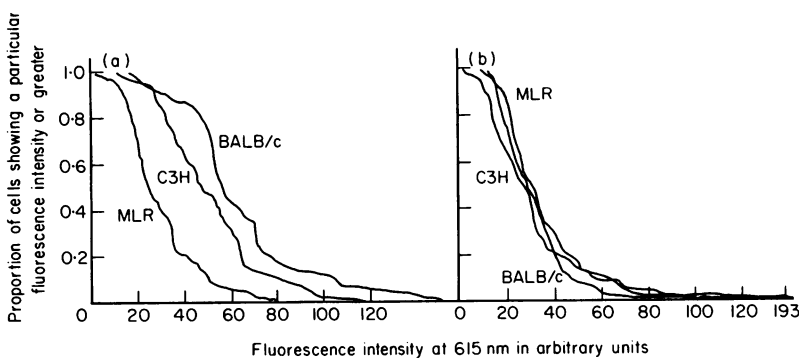

Figure 2 (a) Comparison of fluorescence of BALB/c and C3H mouse thymocytes incubated alone or together in MLR and subsequently labelled with TMRITC. Mean values, BALB/c = 66·1; C3H = 52·9; MLR = 31·1. (b) As above with thymocytes pretreated with sodium azide. Mean values, BALB/c = 31·6; C3H = 31·0; MLR = 36·3 (100 cells counted in each experiment).

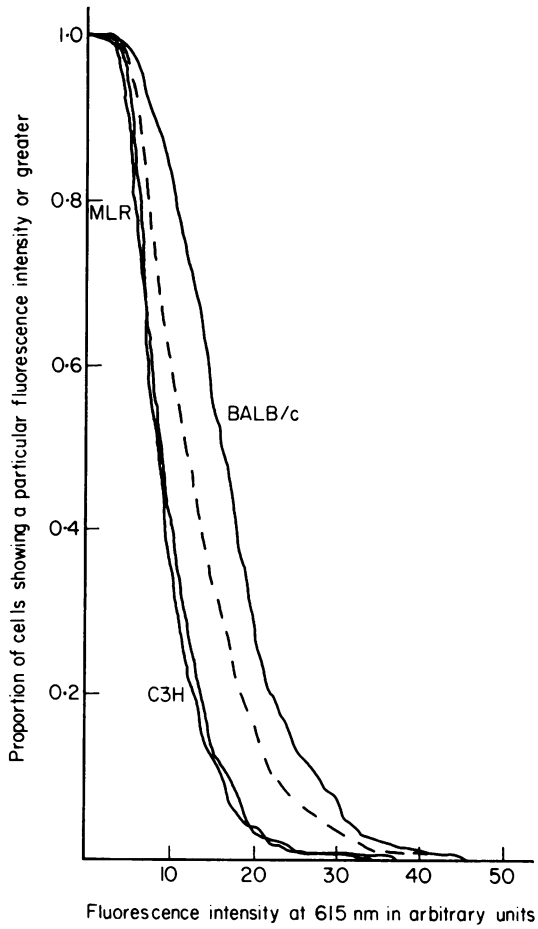

Figure 3. Comparison of fluorescence of BALB/c and C3H mouse thymocytes incubated alone or together in MLR and subsequently labelled with TMRITC. The MLR reaction is significantly ($P<0{\cdot}001$) less than the arithmetical mean position (interrupted curve) between the two individual cell types (500 cells counted in each experiment). Mean values, BALB/c = 16·1; C3H = 8·5; MLR = 8·8.

deviation from the mean fluorescent emission of the two individual cell types (Fig. 2b). The mixed lymphocyte reaction results were not consistently reproducible; in one other experiment, using 3 h incubation, the fluorescent emission from the mixed cells was significantly higher than that from either strain alone ($P<0{\cdot}001$).

DISCUSSION

The experiments show that it is feasible to detect early changes within a few hours of mitogen stimulation of lymphoid cells or in the mixed lymphocyte reaction, by microfluorimetry of cells labelled with TMRITC. A significant change in fluorescent emission can be regularly detected within 3 h of stimulation. The mitogen test has been performed with either pre-stimulation or post-stimulation fluorescent labelling of the cells and both procedures gave much the same result, but pre-labelling is more convenient. Inhibition of the mitogenic response with sodium azide also inhibited the change in fluorescence. We are unable to explain why PHA and Con A stimulation gave increased fluorescent emission, while PWM gave a decrease, except on the basis of chemical and biological differences between the mitogens and physiological differences between their target cells. Some lack of consistency in the mixed lymphocyte reaction results may likewise be attributable to physiological variation between different samples of lymphoid cells. Other fluorescent cell probe methods are also suitable for detecting these reactions, and we have recently reported the use of acridine orange labelling of cell nuclei as an effective procedure for the purpose (Nairn, Rolland, Halliday, Jablonka & Ward, 1978). We have not felt it necessary to compare the present rhodamine probe technique with conventional stimulation tests, e.g. tritiated thymidine incorporation, because these only detect late cellular events.

The molecular site of the TMRITC labelling has not been specially studied but it seems likely that cell membrane molecules are predominantly labelled covalently by the procedure used, and that changes in membrane molecular configuration modify the fluorescent emission of the conjugated molecules. It is a general property of fluorochromes that molecular rigidity and planarity, and the molecular environment, are associated with their fluorescence intensity (Ward & Fothergill, 1976). The fluorescence change observed on mitogen stimulation was small and detectable only by careful attention to experimental detail using sophisticated microfluorimetry and tedious visual microscopic field location. This last disadvantage of manual microfluorimetry should be overcome by use of a flow cytofluorimeter for the assays, and such a field trial of the practical applications of TMRITC cell labelling is required. It would be expected that flow cytofluorimetry, by permitting rapid measurements of much larger numbers of cells, will open the way to detection of fluorescence changes in small populations of stimulated cells as may occur with specific antigen stimulation.

Even without automation, we have shown that a fluorescent cell probe method will permit detection of mitogen activation of lymphoid cells and mixed lymphocyte reactivity within three hours of stimulation. This should eventually lead to time-saving practical applications in the routine immunology laboratory.

ACKNOWLEDGMENTS

This work was supported by grants from the National Health and Medical Research Council and the Anti-Cancer Council of Victoria. We thank Dr E.A. Gehan for initial advice on the statistical assessment of the fluorimetry results.

ADDENDUM

Since going to press, it has been found that the dialysis of the rhodamine-labelled lymphocytes can be limited to 2 h, without loss of reproducibility but presumably with a gain in cell preservation.

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