

Acta Medica Okayama

Volume 35, Issue 5

1981

Article 2

NOVEMBER 1981

A double antibody radioimmunoassay for measurement of IgG, IgA and IgM synthesized by human lymphocytes in vitro.

Taro Asano*

Keisuke Yano[†]

Tadashi Ofuji[‡]

*Okayama University,

[†]Okayama University,

[‡]Okayama University,

A double antibody radioimmunoassay for measurement of IgG, IgA and IgM synthesized by human lymphocytes in vitro.*

Taro Asano, Keisuke Yano, and Tadashi Ofuji

Abstract

To investigate cellular interactions between human T and B lymphocytes in various diseases, we established a technique to prove terminal differentiation of B lymphocytes into immunoglobulin synthesizing and secreting cells. We also established a double antibody radioimmunoassay to measure the amount of IgG, IgA and IgM synthesized and secreted in culture supernatants. Purified immunoglobulins were obtained from sera of patients with myeloma or macroglobulinemia. The peripheral blood lymphocytes from 25 normal individuals had the geometric mean synthetic rates of 1886 ng for IgG, 1607 ng for IgA and 1173 ng for IgM per 1×10^6 cells when cultured for nine days in the presence of pokeweed mitogen. The method is simple and sensitive, and is thought to be useful for examining human lymphocyte function in vitro.

KEYWORDS: lymphocytes, PWM, immunoglobulins, RIA.

*PMID: 6458995 [PubMed - indexed for MEDLINE]

Copyright (C) OKAYAMA UNIVERSITY MEDICAL SCHOOL

Acta Med. Okayama 35, (5), 319—326 (1981)

A DOUBLE ANTIBODY RADIOIMMUNOASSAY FOR MEASUREMENT OF IgG, IgA AND IgM SYNTHESIZED BY HUMAN LYMPHOCYTES *IN VITRO*

Taro ASANO, Keisuke YANO and Tadashi OFUJI

*Third Department of Internal Medicine Okayama University Medical School,
Okayama 700, Japan*

Received May 15, 1981

Abstract. To investigate cellular interactions between human T and B lymphocytes in various diseases, we established a technique to prove terminal differentiation of B lymphocytes into immunoglobulin synthesizing and secreting cells. We also established a double antibody radioimmunoassay to measure the amount of IgG, IgA and IgM synthesized and secreted in culture supernatants. Purified immunoglobulins were obtained from sera of patients with myeloma or macroglobulinemia. The peripheral blood lymphocytes from 25 normal individuals had the geometric mean synthetic rates of 1886 ng for IgG, 1607 ng for IgA and 1173 ng for IgM per 1×10^6 cells when cultured for nine days in the presence of pokeweed mitogen. The method is simple and sensitive, and is thought to be useful for examining human lymphocyte functions *in vitro*.

Key words: lymphocytes, PWM, immunoglobulins, RIA.

Recent investigations(1) have revealed that pokeweed mitogen (PWM) which is a plant lectin has a strong mitogenic effect on human B lymphocytes. B lymphocytes stimulated by PWM start to divide and differentiate into immunoglobulin(Ig) secreting cells in the presence of helper T lymphocytes *in vitro* (2). Suppressor T lymphocytes can inhibit these B lymphocytes from differentiating into Ig secreting cells(3).

Using this system, we are able to investigate the mechanisms of cellular interaction among human B cells, helper T cells and suppressor T cells *in vitro*.

To analyze defects in immunoregulatory mechanisms which are thought to play an important role in the pathogenesis of immunodeficiency diseases and autoimmune diseases such as systemic lupus erythematosus, we developed a PWM induced Ig(s) synthesis system and a specific double antibody radioimmunoassay (RIA) for measurement of IgG, IgA and IgM in culture supernatants.

MATERIALS AND METHODS

Purification of human immunoglobulins. The IgG, IgA and IgM proteins for the standard and for radiolabelling were obtained from the sera of patients with IgG myeloma, IgA myeloma and macroglobulinemia. Initially, gammaglobulin fractions were obtained by 33.3% ammonium

sulfate precipitation for IgG, and 50% saturation for IgA and IgM. IgG was purified by ion exchange column chromatography of DEAE cellulose equilibrated with 0.005M phosphate buffer (PB), pH 8.0. Forty ml of fractions unbound to DEAE cellulose was concentrated to 7 ml by ultrafiltration (DIAFLO; Amicon). The final concentration of IgG was 52.0 mg/ml as determined by the Folin-Lowry method. IgA was also purified by ion exchange chromatography on a DEAE cellulose column. Twenty ml of fractions eluted by 0.02 M Tris PB, pH 8.0 containing 0.1 M NaCl was concentrated to 3 ml. The final concentration of IgA was 10.3 mg/ml. IgM was purified by gel filtration on Sephadex G-200 (Pharmacia) in 0.02 M Tris PB, pH 8.0 containing 0.3 M NaCl. Ten ml of fractions from the early half of the first peak of protein was concentrated to 1 ml and the final concentration was 3.0 mg/ml. Ouchterlony immunodiffusion tests of each purified Ig showed a single band with specific rabbit anti-human Ig serum and no contamination of other classes of Ig.

Radiolabelling of immunoglobulins. Purified IgG, IgA and IgM were radiolabelled with ^{125}I -Na by the chloramine-T method of Greenwood (4). Ten μl of each Ig in 10 μl of phosphate buffered saline (PBS), 10 μl of 0.5 M PB containing 500 μCi ^{125}I -Na and 5 μg of freshly dissolved chloramine-T in 10 μl of PBS were mixed in a small plastic container and allowed to react each other at room temperature (RT) for 30 seconds before the addition of 63 μg of freshly dissolved sodium metabisulfate in a volume of 10 μl of PBS. Each radiolabelled Ig was separated from the free ^{125}I -Na by gel filtration on a Sephadex G-25 column equilibrated with PBS for IgG and IgA, and PB containing 0.3 M NaCl for IgM. To prevent nonspecific binding of radiolabelled Ig(s), 1 ml of PBS containing 1% bovine serum albumin (BSA) was applied to this column first and 0.1 ml of 1% BSA-PBS was added to each collecting tube before running. About 1 ml of eluate was collected into each tube and radioactivity in each fraction was determined by counting 10 μl from each tube for 6 seconds in a gamma ray counter. 80-90% of the radioactivity in the labelled protein preparation was precipitated by a specific anti-human Ig serum.

Antisera. Rabbit antisera specific to IgG, IgA and IgM were obtained commercially (Hoechst, Japan). Goat anti-rabbit gammaglobulin serum and normal rabbit serum (NRS) were also commercially available (Eiken). Rabbit anti-human gammaglobulin sera were diluted to 1/2000 with PBS containing 1% BSA, 0.1% sodium azide and NRS which was diluted to 1/1000-1/2500 to reduce its reactivity to human gammaglobulins. Goat anti-rabbit gammaglobulin sera was also diluted to 1/100-1/200 with 1% BSA-PBS for the same purpose.

Separation and culture of lymphocytes. Lymphocytes were obtained by the Ficoll-Conray gradient technique from defibrinated venous blood. To avoid any contamination of serum protein, cells were washed five times with RPMI-1640. Cells were suspended in medium RPMI-1640 supplemented with 10% fetal calf serum (FCS), Penicillin (PC; 100 $\mu\text{g}/\text{ml}$), Streptomycin (SM; 100 $\mu\text{g}/\text{ml}$) and one ml aliquots containing 1×10^6 cells/ml were placed into 19 \times 125 mm round bottom polypropylene tube (Falcon, # 3033) and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 9 days after adding 10 μl of PWM to the experimental tubes. At the termination of the culture period, each tube was centrifuged at 2000 rpm for 10 min and the supernatant was removed and stored at -20°C until the time of assay.

Measurement of Ig(s) in culture supernatants by RIA. The procedure employed was essentially the same as that for measurement of IgE by Gleich *et al.* (5). All reagents were diluted in PBS pH 7.4 containing 1% BSA and 0.1% sodium azide. Samples in a volume of 0.1 ml, either supernatants to be tested or purified Ig standard, were mixed with properly diluted rabbit anti-human IgG, IgA or IgM serum in the disposable plastic tubes in duplicate and placed at room temperature (RT) for 90 min. Each labelled Ig in a volume of 0.1 ml (approximately

20000 cpm) was added into those tubes and incubated another 90 min at RT. After incubation, diluted goat anti-rabbit gammaglobulin sera in a volume of 0.1 ml was added and incubated over night at 4°C to allow these reagents to react fully. Next day the precipitates were centrifuged at 3000 rpm for 30 min and recentrifuged for another 5 min at RT to make them stick tightly to the bottom. Supernatants was decanted and the radioactivity in the precipitates were determined in a gamma scintillation counter. To determine the counts non-specifically trapped in the precipitates, the same procedure was followed without adding antisera specific to human gammaglobulins. These counts averaged less than 10% of total counts. The percent inhibition was calculated according to the following formula;

$$\% \text{ inhibition} = \left(1 - \frac{\text{counts of standard or sample} - \text{non-specific counts}}{\text{counts of specific binding} - \text{non-specific counts}}\right) \times 100$$

A standard inhibition curve was prepared for each assay covering the range from 10 to 5000 ng/ml and the concentration of Ig(s) was read from this standard curve.

RESULTS

The specificity of the assay system. The results of a typical IgG RIA are shown in Fig. 1. Addition of 10 ng of purified IgG per tube produced significant inhibition and the range extended to 5000 ng per tube which inhibited almost 90% of specific counts. To test both the specificity of this assay and the contamination of

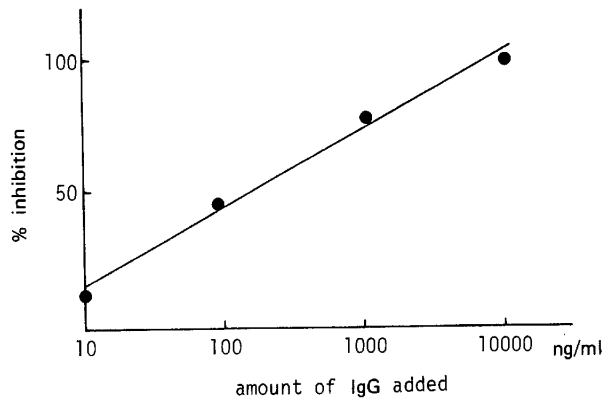


Fig. 1. Standard curve of IgG RIA. Addition of 10 ng of purified IgG inhibit its specific binding significantly. The more IgG was added, the higher inhibition was obtained. 5000 ng of IgG inhibited almost 90% of specific counts.

TABLE 1. SPECIFICITY OF ASSAY SYSTEM*

Amount of Ig added (ng/ml)	% Inhibition		
	IgG	IgA	IgM
10	13.2	n.d.	n.d.
100	44.2	n.d.	n.d.
1000	78.3	n.d.	n.d.
10000	100.0	9.5	17.2

* The data of IgG and anti-IgG system are presented as the representative assay.

n.d. : not done

different classes of Ig, purified IgA or IgM was added into the reaction mixture consisting of radiolabelled IgG and specific antibody, IgG or IgM into IgA assay tubes and IgG or IgA into IgM assay tubes. As is presented in Table 1, addition of 10000 ng of purified IgA or IgM to the mixture of IgG and anti-IgG sera caused 9.5% or 17.2% inhibition of specific binding. This means that 10000 ng of purified IgA or IgM contains less than 10 ng or 12 ng of IgG, respectively. These results indicated that the different class of Ig, IgA and IgM, did not inhibit the specific binding of labelled IgG to anti-IgG antisera. Each purified Ig had at most 0.2% contamination by other classes of Ig.

Declining curves of radioactivity in the precipitates of labelled Ig and specific antisera. It is known that the long time storage of labelled Ig results in loss of both radioactivity and reactivity to specific antisera. Fig. 2 shows the radioactivities in the precipitates of labelled Ig and anti-Ig antisera at different intervals after radiolabelling. These data demonstrated that radiolabelled IgG and IgA were stable but that labelled IgM was prone to decay. This fragility of labelled IgM may rest on its short life span and higher catabolic rate. To make this RIA accurate, we did not use the decayed labelled Ig of which specific radioactivities decreased to less than 40% of the initial activity. Generally, radiolabelled Ig was still usable 3 weeks after from its radiolabelling.

Kinetic curves of Ig(s) production of normal lymphocytes. To be Ig producing cells, lymphocytes require information from mitogen and different type of lymphocytes such as helper T cells before they can differentiate into Ig secreting cells. Because of requirement of these steps, it takes certain periods of time for them before Ig can be detected in culture supernatants. We measured the levels of secreted Ig in the supernatants on day 3, 5, 7, 9 and 11 after PWM stimulation. The kinetic curve of IgG synthesis is shown in Fig. 3. There was very little production during first four days of culture. However, the amount of IgG increased logarithmically

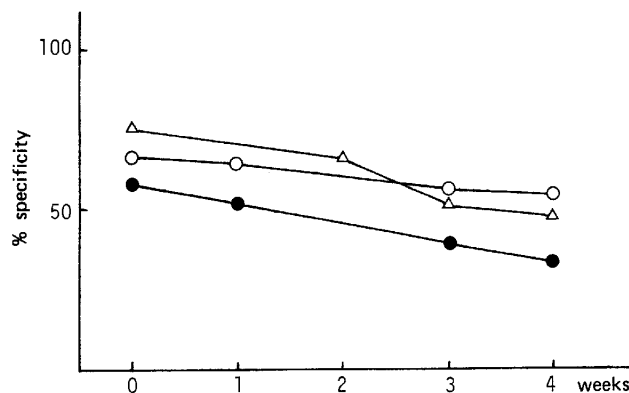


Fig. 2. Declining curve of radioactivity of ^{125}I -labelled Ig. ^{125}I -labelled IgM (●—●) lost the radioactivities in the precipitates very rapidly but both labelled IgG (○—○) and IgA (△—△) were still stable 4 weeks after their labelling.

after 5 days of culture and reached a plateau around the 9th day of culture. The determination of synthesized Ig was done using the 9 day culture supernatants, thereafter.

Stimulative activities of FCS. In a certain experiment, we could detect Ig in the supernatant of normal lymphocyte culture without PWM. This suggested the possibility of stimulative activity existing in the FCS used. Therefore, normal lymphocytes were cultured using medium supplemented with 10 different lots of FCS and the amounts of Ig secreted without PWM were measured. The results are shown in Table 2. The number 5 FCS had the least stimulative effect

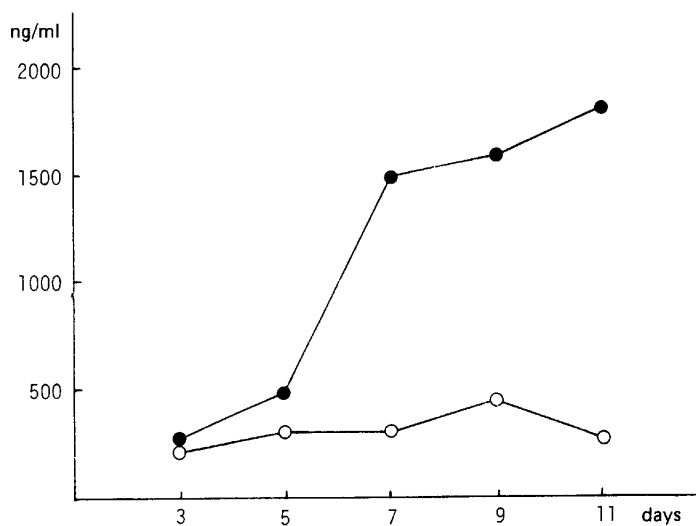


Fig. 3. Kinetic curves of IgG synthesis. Normal lymphocytes could produced little amount of IgG without PWM (○—○) at any culture periods. Addition of PWM (●—●) increased IgG production after 5th day of culture.

TABLE 2. STIMULATIVE ACTIVITIES OF DIFFERENT LOTS OF FCS

FCS No.	Produced IgG (ng/ml)		
	Donor A	Donor B	Donor C
1	> 5000	3000	n.d.
2	> 5000	2000	n.d.
3	> 5000	1650	n.d.
4	> 5000	4700	n.d.
5	1700	n.d.	50
6	> 5000	n.d.	460
7	> 5000	n.d.	3300
8	> 5000	n.d.	> 5000
9	> 5000	n.d.	3500
10	> 5000	n.d.	3700

n.d. : not done

on IgG synthesis. Therefore, this FCS was used throughout all the experiments.

Concentration of IgG, IgA and IgM in culture supernatants. The levels of Ig(s) in culture supernatants of lymphocytes from 25 normal individuals are shown in Fig. 4. Normal lymphocytes could synthesize significant amount of Ig after PWM stimulation. The geometric mean values of IgG, IgA and IgM synthesis were 360, 390 and 298 ng per 1×10^6 cells respectively, after the nine days culture without PWM stimulation. Ig synthesis was markedly elevated when PWM was added at the initiation of culture. As indicated in Fig. 4, the geometric mean rates of IgG, IgA and IgM synthesis were 1886, 1607 and 1173 ng per 1×10^6 cells, respectively, over the nine days culture periods.

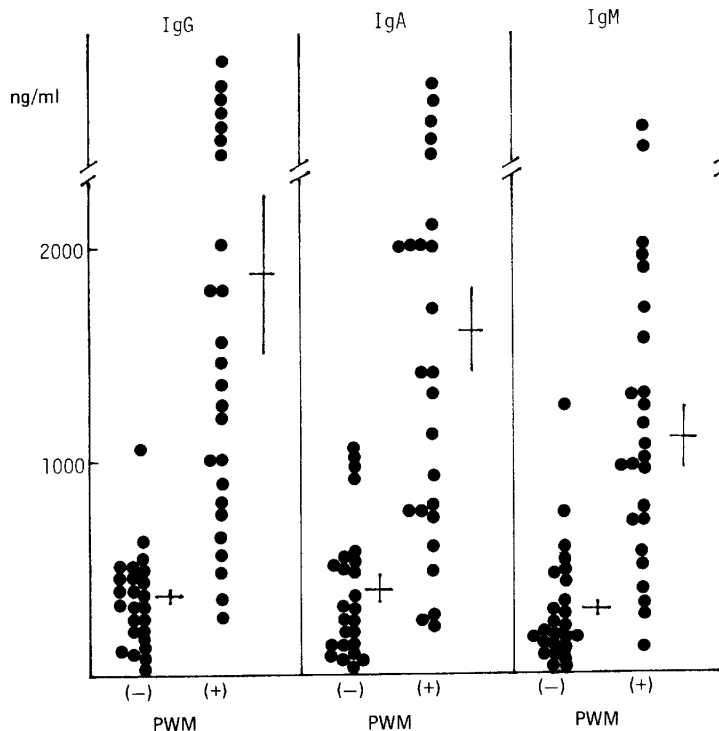


Fig. 4. Amounts of Ig (s) synthesized by 1×10^6 lymphocytes. As was expected, addition of PWM at the beginning of culture stimulated lymphocytes to synthesize a large amount of each Ig class. Mean \pm S.E.M. are shown.

DISCUSSION

In this study, we have established and utilized a sensitive double antibody RIA to measure IgG, IgA and IgM synthesized and secreted into culture supernatants by human lymphocytes. This procedure requires only minute quantities of purified Ig and specific antisera. Ten μg of purified Ig was enough for radiolabeling and rabbit anti-human gammaglobulin sera could be diluted to 1/2000. The

method is sensitive and able to detect as little as 10 ng per ml of Ig. Its relative simplicity permits to test a large number of samples to be tested at one time.

The critical points of this assay are extensive washing of lymphocytes to avoid the contamination of serum protein carried over, and selection of FCS which should have no stimulative activity on lymphocytes leading to Ig production. We washed lymphocytes 5 times, because lymphocytes washed three times carried serum Ig over into the culture system and more than 5000 ng per ml of IgG was detected even at the initiation of culture. The most stimulative FCS stimulated lymphocytes to synthesize a great amount of Ig(s) without PWM stimulation (Table, 2). In either case, it is difficult to estimate the responsiveness of lymphocytes to PWM.

The peripheral blood lymphocytes from 25 normal individuals produced significant amount of Ig(s). Their geometric means of the rate of synthesis were 1886 ng for IgG, 1607 ng for IgA and 1173 ng for IgM when cultured for 9 days in the presence of PWM. These results were comparable to the values reported by Waldmann *et al.* (3). In their studies, the geometric mean rates of synthesis for IgG, IgA and IgM were 1641 ng, 1698 ng and 3715 ng per 2×10^6 cells over the 7 day culture periods. The kinetic curve of Ig synthesis after PWM stimulation shows that Ig(s) detectable in culture supernatants increased rapidly after the 5th day of culture. This indicates that several steps of cellular interaction may be required for maturation of non-Ig-secreting B cells into Ig synthesizing and secreting cells by PWM stimulation.

The sensitivity of the RIA for Ig(s) permits the measurement of Ig in the supernatants of lymphocyte cultures. Therefore, the method is very valuable for studying *in vitro* the function of B cells from patients with various diseases and from normal individuals. Moreover, we can use this assay to assess the function of helper T cells or suppressor T cells, because it has been accepted that the control of Ig synthesis and secretion of PWM simulated B cells requires the cooperation of helper T, suppressor T cells and macrophages (6, 7).

Acknowledgment. The authors wish to thank Dr. Y. Namba, Department of Internal Medicine 3, for his kind gift of sera from patients with myeloma and macroglobulinemia.

REFERENCES

1. Keightley, R.G., Cooper, M.D. and Lawton, A.R.: The T cell dependence of B cell differentiation induced by pokeweed mitogen. *J. Immunol.* **117**, 1538-1544, 1976.
2. Saxon, A., Stevens, R.H. and Ashman, R.F.: Regulation of immunoglobulin production in human peripheral blood leukocytes: cellular interactions. *J. Immunol.* **118**, 1872-1879, 1977.
3. Waldmann, T.A., Broder, S., Blaese, R.M., Durm, M., Blackman, M. and Strober, W.: Role of suppressor T-cells in pathogenesis of common variable hypogammaglobulinemia. *Lancet* **2**, 609-613, 1974.

4. Greenwood, F.C., Hunter, W.M. and Glover, J.S.: The preparation of ¹³¹I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89**, 114-123, 1963.
5. Gleich, G.J., Averbek, A.K. and Swedlung, H.A.: Measurement of IgE in normal and allergic serum by radioimmunoassay. *J. Lab. Clin. Med.* **77**, 690-698, 1971.
6. Rosenberg, S.A. and Lipsky, P.E.: Monocyte dependence of pokeweed mitogen-induced differentiation of immunoglobulin secreting cells from human peripheral blood mononuclear cells. *J. Immunol.* **122**, 926-931, 1979.
7. Fauci, A.S., Pratt, K.R.K and Whalen, G.: Activation of human B lymphocytes II. Cellular interactions in the PFC response of human tonsillar and peripheral blood B lymphocytes to polyclonal activation by pokeweed mitogen. *J. Immunol.* **117**, 2100-2104, 1976.