

Research article

Plaque Forming Cell Technique for Counting IgG and IgM antibody-secreting cells in peripheral blood of patients with rheumatoid arthritis

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ABSTRACT

Antibody Secreting cells are playing a central role in pathogenesis of several autoimmune diseases especially in rheumatoid arthritis. There are few reports describe a method can estimate the number of antibody secreting cells in vitro spontaneously. Here we modified the classical method of plaque forming assay to study the number of IgG and IgM secreting cells [Plaque forming cells (PFCs)] without external stimulators in vitro in peripheral blood of patients with rheumatoid arthritis. Moreover, the relationship between the IgG and IgM concentrations and number of PFC-IgG and IgM (without external stimulators) was studied. The significant increase of IgG as well as IgM antibody secreting cells [plaque forming cell (PFC) - IgG and IgM] was seen in peripheral blood of patients with rheumatoid arthritis ($P < 0.001$) as compared with healthy control group. These findings were associated with high level of IgG and IgM concentration in peripheral blood of patients. The current present study besides providing a modified method to detect IgG or IgM secreting cells (PFC) in vitro, it also proved that the high level of IgG and IgM in peripheral blood associated with spontaneous activity of B-cells.

Keywords: B-lymphocytes, Plaque Forming cells, Spontaneous activation, modification method.

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INTRODUCTION

The B cell hyperactivity caused spontaneous hyper-secretion of antibodies and class switching to different classes of immunoglobulin in the absence of self antigen, and this persisted as an autoimmune phenomenon [1]. Hyper-secretion of antibodies is known as one of an important finding of rheumatoid

arthritis [2]. Overstated class switching was also unaffected by antigen in vitro. Spontaneous activation of hyperactive B cells leads to isotype switching and the development of high titers of immunoglobulin autoantibodies against intracellular proteins [3]. The number of antibody-producing cells



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can be quantified by plaque-forming cell (PFC) assay. The methods of Cunningham-Szenberg and the Jerne-Nordin techniques employ specially prepared slide chambers, in which the antibody-producing B cells are mixed with complement and indicator sheep red blood cells (SRBC), or with trinitrophenol-modified SRBC (TNP-SRBC), with subsequent lysis and counting of plaques. Because IgM antibodies fix complement efficiently, whereas IgG and IgA antibodies do not, unmodified PFC assays measure only IgM antibodies [3]. Another method of measuring the number of antibody-producing B cells (in a class-specific fashion) is to use the ELISPOT technique. The resting B cells used in these procedures are prepared as described in the final support protocols for Percoll gradient centrifugation [4, 5]. In current study the method of Jerne-Nordin was developed to establish a modified technique help to study the number of B cells produce IgG and IgM in vitro without stimulating with any external stimulating factors.

MATERIALS AND METHODS

Patients' samples

Peripheral blood was obtained from 20 RA patients and from 18 age and sex-matched healthy controls. 2010 Rheumatoid arthritis classification criteria were followed by rheumat-ologist in Baghdad specialist hospital and central public health laboratories, Baghdad, Iraq to diagnose the patients with RA [6]. They had never received disease modifying drugs or corticost-eroids for one-month prior time point of present study. Among RA patients there were three male and 17 female, 10 (50%) tested positive for IgM rheumatoid factor, and 12 (60%) tested positive for anti-CCP antibodies; their age was 51.2 ± 10.9 years (mean + SD), and disease activity score-28 (DAS 28) [7] was 3.95 ± 1.12 (mean $\pm$ SD). Nine

anti-CCP positive RA patients donated blood for a second time, ten months after the initiation of treatment with oral methotrexate (MTX) and low-dose prednisone. At the time of experiments, prednisone had been discontinued in all except for seven patients, who were taking 2.5 mg daily, and all of them were taking oral MTX at doses of 10 to 20 mg. The study was conducted following approval from the human ethics committee of ministry of health, Baghdad, Iraq.

Erythrocyte sedimentation rate (ESR) was measured according to standard Westergren techniques. C-reactive protein (CRP) (Latex Test Kit, Diagnostic automation, Inc, USA) was measured in serum that had been frozen and stored at -20°C for less than 4 days. Human IgG ELISA kit and human IgM ELISA kit (Bethyl Laboratories, Inc.) were used to measure the concentrations of essential immunoglobulin (IgM and IgG) in sera of patients and control groups (table 1).

Measurement of essential Immunoglobulins (Igs)

Single radial immunodiffusion method (Mancini) was used to measure the concentrations of essential immunoglobulins (IgM and IgG) in sera of patients and control groups. The manufacture's instruction of Sanofi Diagnostic Pasteur was followed to check the concentrations of these immunoglobulins.

Peripheral blood lymphocytes (PBLs) isolation

Mononuclear cells were separated from fresh heparinized venous blood by a modification of the Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals Inc., New York) buoyant density gradient method of Boyum [8], as previously described [9]. The viability of mononuclear cells was determined by trypan blue exclusion test [10]. The viable cells were adjusted to 10^6 cells/ml by hank balance salt solution (HBSS).

Table 1. Clinical and laboratories finding of patients with RA and healthy control groups.

	RA	Control	P value*
Number of cases	20	18	P>0.05
Mean of age (year)	47.3	45.7	P>0.05
Range of age	33-51	31-54	P>0.05
Female (n and %)	17 (85 %)	15 (83.3 %)	P>0.05
Male (n and %)	3 (15 %)	3 (16.67 %)	P>0.05
Female/male ratio	5.6	5	P>0.05
IgG [mg (100 ml)-1]	2790 ± 743	1510 ± 320	P<0.05
IgM [mg (100 ml)-1]	442 ± 85	123 ± 27	P<0.05
CRP (n and %) (>3 mg l-1)	10 (50 %)	0	P<0.05
IgM rheumatoid factor (n and %)	17 (85 %)	0	P<0.05
ESR (mmh-1)	52.5 ± 12.3 mm	6.4 ± 2.7 mm	P<0.05
Anti-CCP antibodies (n and %)	12 (60 %)		

n, number of case; *, Student's t test for continuous variables, and X2 test for categorical variables.

Preparation of sensitized sheep red blood cells

The standard method of Golstein and Gomperts, (1975) was followed to bind Rabbit anti-(human)-IgG (Sigma), Rabbit anti-(human)-IgM (Sigma) on sheep red blood cells (SRBCs) [11].

Antibody plaque-forming cell assay

The number of plaque-forming cells (PFCs) was determined using our modification of the Jerne plaque assay [3]. Five test tubes were used in present experiment. 0.1 ml of SRBCs (3%) coated with rabbit anti-(human)-IgG was added to first and second test tube. In third and fourth test tubes, 0.1 ml of SRBCs coated with rabbit anti-(human) IgM was added. Fifth test tube containing 0.1 ml of uncoated SRBCs (5%). 0.1 ml of 5×10^6 /ml [HBSS containing 0.5% w/v gelatin (Difco, Bacto)] of peripheral blood lymphocyte (PBL) was added to first, third and fifth test tubes. 2 ml of 0.75 % of agarose L [prepared in Eagle medium pH 7.2 (Flow Lab.)] was added to each test tube. The test tubes containing aliquots of mixture was maintained at 40 °C in a water bath. The mixture spread quickly onto warmed Petri dishes (37 °C) to form a thin layer. Plates were incubated for 2 h at 37 °C. 2 ml of complement inactivated goat IgM anti-(human)-IgG (1:500) (sigma) was added to first, second and fifth plates. The plates were washed three times with PBS (pH 7.2, 0.1M) after incubation for 1 h at 37 °C. 2 ml of genia pig serum (1:10 v:v serum:PBS) was added onto each plate and then the plates were incubated for 30 min at 37 °C. The plates were fixed by glutaraldehyde (0.25% PBS) and then PFCs were counted. Results were reported as PFCs/million cells. The experiments were conducted three times.

Statistical analysis

All values have been used to give a mean value and the standard deviation calculated. The correlation coefficient test was used to check the relationship between to groups. The differences were analyzed by using Student's t-test employing Origin version 8.0 software. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Plaque forming cells in peripheral blood of patients with AICAH

Fig. 1a shows the plaque forming unite which composing from plaque forming cells (antibody

secreting cell) surrounding by clear zone, while Fig. 1b presents the SRBCs mixed with peripheral blood lymphocytes, no PFC appeared in this figure as the lymphocytes in this place did not produce antibody attach to SRBCs [coated with either rabbit anti-(human)-IgG or rabbit anti-(human)-IgM], that is why; no clear zone produced in this area. The number of plaque forming unites almost equal to number of plaque forming cells (PFCs).

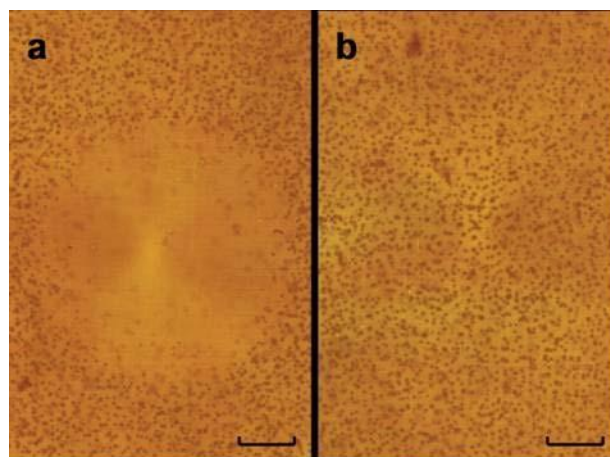


Fig 1. Plaque forming unite composing of plaque forming cell (PFC) in center surrounding by clear zone (a). b, low power of SRBCs mixed with PBL, no plaque was seen (bars, 200 μ m).

To check whether the hemolytic plaques were specific for B-cells which produce either IgG or IgM, two technical controls were included. First, plates composed of uncoated SRBCs mixed with PBL collected from patients and second composed of coated SRBCs with rabbit anti-(human)-IgG or rabbit anti-(human)-IgM but mixed with PBS. In both case of technical control no plaques were observed. However, when coated SRBCs [rabbit anti-(human)-IgG or rabbit anti-(human)-IgM] mixed with PBLs collected from patients or health volunteers, high number of plaques were yielded (Fig 2a). In present study, the significant elevation in number of PFC-IgG and PFC-IgM in patients suffering from rheumatoid arthritis as compared with healthy control group was seen confirming high number of B-cells that produce IgG and IgM without exposing to external stimulators in peripheral blood of patients with rheumatoid arthritis. On the same hand, the significant increase ($P < 0.005$) in concentration of IgG and IgM in peripheral blood of patients with

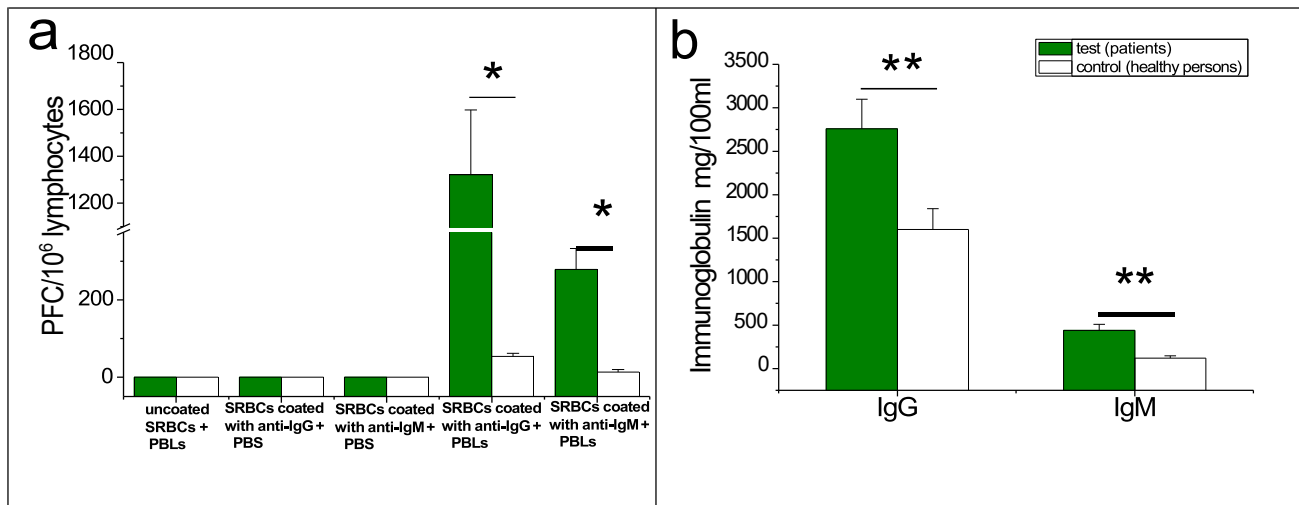


Fig. 2. Plaque forming cells (PFCs) of IgG and IgM (a), and Immunoglobulins (IgG and IgM) concentration (b) in peripheral blood of patients with rheumatoid arthritis and healthy control volunteers. The plaques were seen only in case of mixture of coated SRBCs with rabbit anti-(human)-IgG or rabbit anti-(human)-IgM and PBLs for patients and healthy control. Number of PFC-IgG and PFC-IgM in peripheral blood of patients with rheumatoid arthritis was significantly higher than number of these plaques in peripheral blood of healthy control persons ($P < 0.001$). Similarly, the concentration of IgG and IgM in peripheral blood of patients was significantly higher than control ($P < 0.005$). Asterisks indicate a significant difference from the healthy control group (*, $P < 0.001$); **, $P < 0.005$).

rheumatoid arthritis as compare with healthy control group was observed (Fig. 2b). Fig. 3 shows the relationship between the number of PFCs and Immunoglobulin (IgG and IgM) levels in peripheral blood of patients with rheumatoid arthritis. The positive relationship was found between the level of IgG and PFC-IgG in peripheral blood of patients with rheumatoid arthritis. Similar trend was seen when the relationship was checked between the concentration of IgM and number of PFC-IgM in peripheral blood of patients with rheumatoid arthritis. No relationship was observed between the number of PFCs and concentrations of Immunoglobulin in peripheral blood of healthy control group. This study confirmed the high level of Immunoglobulin belonging to high activity of B-cells that producing Immunoglobulin without external stimulators.

DISCUSSION

Plaque forming cell technique commonly used to check the number of antibody secreting cell in peripheral blood or in spleen (12-17). Most of previous studies checked the number of antibody secreting cells (PFCs) against sheep red blood cells after injecting animal with particular number of SRBCs. These previous techniques help researchers to estimate the number of PFCs which produce total essential immunoglobulin in peripheral

blood or in spleen. In case of estimation of PFCs in human body another modified technique presented by Sedgwick et al. (1983) used ELISA plates coated with anti-IgM or -IgG Abs or calf thymus ssDNA or dsDNA (18, 5). In current study, the method of Jerne-Nordin technique was modified, here the SRBCs were coated with either rabbit anti-(human)-IgG or rabbit anti-(human)-IgM which prepared in rabbit. The IgG or IgM produced by antibody secreting cells (PFCs) attached with anti-IgG or anti-IgM on SRBCs. The yielded immune-complexes activated the added complement and ultimately the clear plaques produced around the IgG or IgM secreting cells (PFC-IgG or PFC-IgM). In this technique no B lymphocytes stimulators were added. That is why, it can be jugged that these cells are plasma cells produce antibodies spontaneously. This study provided a simple and cheap technique that help to detect IgG or IgM antibody secreting cells spontaneously in vitro. Antibody producing cells play an important role in pathogenesis of different autoimmune diseases such as rheumatoid arthritis, autoimmune hepatitis and systemic lupus erthematosus (19-21). In previous autoimmune diseases the extra production of antibodies are related with the pathogenesis of autoimmune diseases (22-24). That is why, some drugs, which

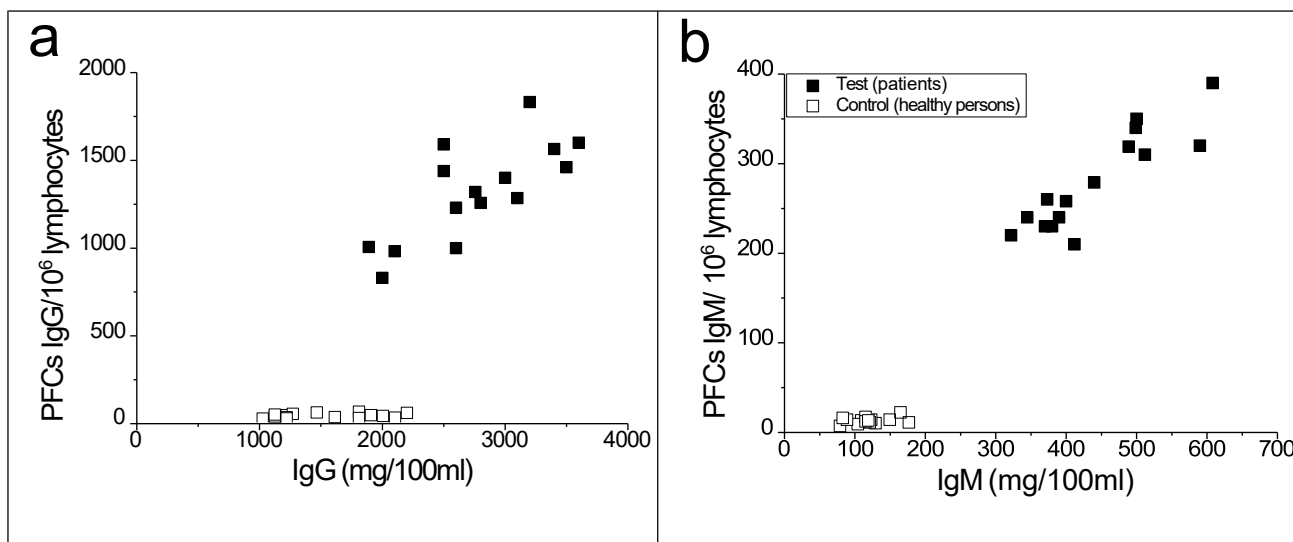


Fig 3 (a), Relationship between number of PFC-IgG and concentration of IgG. (b), Relationship between the number of PFC-IgM and concentration of IgM (b) in peripheral blood of patients with rheumatoid arthritis and healthy control volunteers. Positive relationship was observed between PFC-IgG and IgG concentration (correlation coefficient= +0.75), also positive relationship was seen between PFC-IgM and IgM concentration (correlation coefficient = +0.89).

reduce the activity of plasma cells to produce antibody is very effective in treatment of several autoimmune diseases (25). In present study we also found a positive relationship between the level of essential immunoglobulins and number of antibody secreting cells (PFCs) which produced antibody in vitro without external stimulators. Current study besides providing a modified technique to estimate the number of antibody secreting cells (IgG or IgM) we confirmed the relationship between the spontaneous activation of B-cells and the level of immunoglobulin in peripheral blood of patients with rheumatoid arthritis.

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Conflicts of Interest

We have no financial interests related to the material in the manuscript.

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