Volume 333, number 1,2, 35–38 FEBS 13131 October 1993

Association constants of anti-hapten monoclonal IgG1 with mouse FcγRII in the presence and absence of hapten

Martina T. Sumner*

Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3290, USA

Received 19 August 1993

The binding of five different mouse monoclonal anti-dinitrophenyl (DNP) IgG1 monomers, in the presence and absence of DNP-glycine, to FcγRII on the mouse macrophage-like cell line J774 has been investigated. Membrane association constants were estimated using a competitive radioimmunoassay in which the ability of the IgG1 monomers to block the binding of ^{125}I-labelled Fabs of the anti-FcγRII monoclonal antibody 2.4G2 was monitored. The IgG1–FcγRII association constants ranged from $(8 \pm 1) \times 10^4$ M^{-1} to $(6 \pm 2) \times 10^5$ M^{-1}. No large differences in the IgG1–FcγRII association constants were measured for different IgG1 antibodies or for any single IgG1 antibody in the presence and absence of DNP-glycine.

Fc receptor; Cell surface antigen; Anti-(Fc receptor) antibody; Macrophage; Cell surface binding equilibrium; Radioimmunoassay

1. INTRODUCTION

Although a body of evidence shows that major conformational changes do not occur in immunoglobulins upon hapten or antigen binding [1], the possibility that occupation of the antigen binding regions causes changes in Fc region effector functions, such as Fc receptor binding and complement activation, has not been completely ruled out [2–6]. In addition, previous investigations have often used either polyclonal IgG or only one monoclonal antibody of a given IgG subclass to examine the association constants of IgG–FcγRII interactions [7–11], but the affinity of Fc regions for Fc receptors might, in theory, differ somewhat between different antibodies within a given subclass. In this work, potential differences in the equilibrium association constants for different monoclonal IgG1 anti-dinitrophenyl (DNP) antibodies with a mouse Fc receptor, FcγRII [7,12], were investigated. The effects of the occupation of the hapten binding regions on the IgG1–FcγRII association constants were also examined. Association constants for the mouse IgG1 antibodies were measured on cells of the macrophage-related line J774 using a competitive radioimmune assay with ^{125}I-labelled Fabs of the anti-FcγRII monoclonal antibody 2.4G2 [13]. A preliminary report has appeared in abstract form [14].

*Corresponding author. *Present address:* Laboratory of Cellular and Molecular Pharmacology, National Institute of Environmental Health Sciences, P.O. Box 12233 (MD 7-10), Research Triangle Park, NC 27709, USA. Fax (1) (919) 541-1898.

2. MATERIALS AND METHODS

2.1. *Cells*

J774A.1, a macrophage-related cell line which expresses FcγRII, was obtained from the University of North Carolina Tissue Culture Facility. 2.4G2, a rat-mouse hybridoma which secretes antibodies specific for FcγRII [13], was a gift from B. Diamond of the Albert Einstein College of Medicine. Five different hybridomas which produce anti-DNP IgG1 were obtained as follows: ANO2, ANO5, and ANO6, H.M. McConnell, Stanford University [15,16]; 1B7.11, American Type Culture Collection; and DHK109.3, N.R. Klinman, Scripps Clinic and Research Foundation [17]. J774 and hybridoma cells were maintained in media supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU penicillin, 100 μg/ml streptomycin, and fetal calf serum (FCS) that had been heat-inactivated for 30 min at 56°C, as follows: J774, ANO2, and 1B7.11, DMEM/F12, 5% FCS; ANO5, RPMI 1640, 2.5% FCS; ANO6 and DHK109.3, RPMI 1640, 1% FCS.

2.2. *Antibodies*

Mouse IgG, rat IgG Fab, and mouse IgG F(ab)′$_2$ were obtained commercially (Jackson ImmunResearch, West Grove, PA). 2.4G2 antibodies were purified from cell supernatants by affinity chromatography using an anti-(rat immunoglobylin κ light chain) antibody (MAR 18.5), and 2.4G2 Fabs were produced and purified, as described [18]. Anti-DNP IgG1 were isolated from cell supernatants by DNP-(human serum albumin) affinity chromatography [19]. Antibody isotypes were confirmed using the EIA Grade Mouse Typer System (Bio-Rad, Richmond, CA). All antibodies were clarified for 30 min at $134{,}000 \times g$ before use. Gel filtration [20] showed that the antibodies eluted with symmetrical peaks corresponding to IgG monomers. IgG1 concentrations were determined by spectrophotometry ($\varepsilon_{280} = 1.4$ $ml \cdot mg^{-1} \cdot cm^{-1}$). Antibodies were labelled with ^{125}I (New England Nuclear, Wilmington, DE) using iodo-beads (Pierce Chemical, Rockford, IL). 80–95% of the ^{125}I in chromatographed protein solutions was shown to be precipitated by trichloracetic acid. Specific activities were about 5×10^5 cpm/μg. IgG oligomers were formed by crosslinking solutions at 0.5 mg/ml in phosphate-buffered saline (PBS; 0.05 M sodium phosphate, 0.15 M sodium chloride, 0.01% sodium azide, pH 7.4) with an 800-fold molar excess of disuccinimidylsuberate (DSS) (Pierce) for 1 h at 25°C, followed by chromatography on Sephadex G-25 in PBS and SDS-PAGE to confirm oligomer formation. The

association constants for IgG1 antibodies and DNP-glycine (DNP-G) were measured by ultraviolet fluorescence quenching [19].

2.3. *Binding assays*

Aliquots of 10^6 J774 cells in duplicate were washed with 1.5 ml medium (DMEM/F12, 1% FCS, 0.01% NaN_3) and resuspended in 200 μl PBS containing ^{125}I-labelled antibodies, 1.5 mg/ml BSA/PBS, and in some cases unlabelled antibodies and/or DNP-G. For direct binding measurements, the solutions contained [^{125}I]-(2.4G2 Fab), [^{125}I]-(2.4G2), [^{125}I]-(rat IgG Fab), [^{125}I](-DSS-IgG); for inhibition measurements, the solutions contained an ^{125}I-labelled protein and either mouse IgG, anti-DNP IgG1, 2.4G2 Fab, or mouse IgG F(ab)'$_2$ with or without DNP-G (concentrations as specified). After reaction for various times (as noted), cells were pelleted, 50 μl supernatant was reserved for later analysis, and then cells were washed three times in 1.5 ml medium (twice without, and once with resuspension). Centrifugations were at $15{,}000 \times g$ for 1–2 min. Assays were carried out at 0–4°C. Radioactivities were measured in a liquid scintillation counter (Packard TriCarb 2000), Downers Grove, IL) using ScintiVerse Bio-HP fluid (Fisher Scientific, Springfield, NJ).

2.4. *Data analysis*

Binding curves were fit to theoretical forms using ASYST software (Rochester, NY). All experimental uncertainties are standard deviations. Data of the type shown in Figs. 1 through 3 were analyzed as described [21].

3. RESULTS

3.1. *[^{125}I]-(2.4G2 Fab) and [^{125}I]-(2.4G2) binding to J774 cells*

Measurements of the time dependence of [^{125}I]-(2.4G2 Fab) binding to J774 cells indicated that apparent equilibrium was reached after about 20 min, consistent with previous work [22]. Subsequent assays were carried out by allowing [^{125}I]-(2.4G2 Fab) to equilibrate with J774 cells for one hour. Comparison of the radioactivities of solutions equilibrated with cell suspensions to those of calibrated [^{125}I]-(2.4G2 Fab) solutions indicated that the J774 cells depleted the solution concentration of [^{125}I]-(2.4G2 Fab) by 15–85%; thus, the concentration of free [^{125}I]-(2.4G2 Fab) was subsequently obtained from the measured radioactivities of cell supernatants.

Cell pellet radioactivities indicated that [^{125}I]-(2.4G2 Fab) bound to J774 cells in a saturable manner (Fig. 1). Averaging the best-fit values obtained from five independently measured binding curves gave an association constant (K_f) of $(1.5 \pm 0.5) \times 10^9$ M^{-1}. The concentration of bound [^{125}I]-(2.4G2 Fab) at saturation is 5.4 ± 1.3 nM (Q), which corresponds to $(6.5 \pm 1.6) \times 10^5$ FcγRII/cell. These values for K_f and Q agree well with previous measurements [7,23]. Failure to account for the cellular depletion of the solution of [^{125}I]-(2.4G2 Fab) gave association constants that were artifactually low by a factor of ten.

The best fits of two independently obtained binding curves for intact [^{125}I]-(2.4G2) to the functional form for a reversible bimolecular reaction gave an association constant of $(4.1 \pm 2.0) \times 10^9$ M^{-1} and a saturating density of $(3.7 \pm 0.4) \times 10^5$ molecules/cell. This value for K_f is only slightly larger than the measured constant for

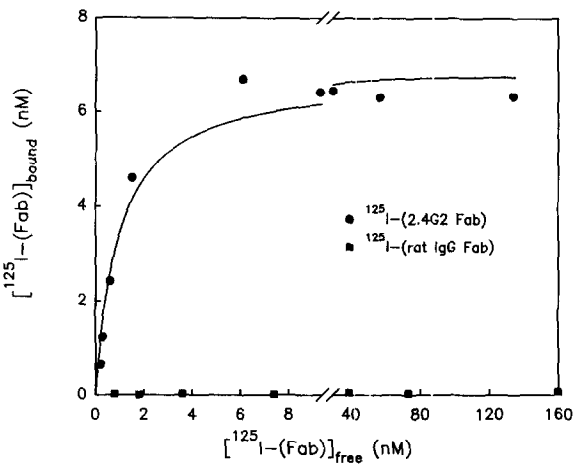

Fig. 1. [^{125}I]-(2.4G2 Fab) binding to J774 cells. For cells treated with various concentrations of [^{125}I]-(2.4G2 Fab) for 1 h, cell pellet radioactivities approached saturation at concentrations $\geq$ 10 nM and were very low for [^{125}I]-(rat IgG Fab). The association constant of this curve is 10^9 M^{-1}.

[^{125}I]-(2.4G2 Fab), suggesting that a fraction of the bound [^{125}I]-(2.4G2 IgG) may not have bound to the cell surface in a bivalent manner. Previous work has yielded similar association constants for intact 2.4G2, 2.4G2 F(ab)'$_2$ and 2.4G2 Fab on J774 surfaces [13,24].

The specificity of [^{125}I]-(2.4G2 Fab) binding was confirmed in that the cell pellet radioactivity was reduced 50-fold for [^{125}I]-(rat IgG Fab) (160 nM, 1 h) (Fig. 1). In addition, a 100-fold excess of unlabelled 2.4G2 Fab reduced [^{125}I]-(2.4G2 Fab) binding 45-fold (200 nM, 1 h), and the measured association constant (K_f) did not change for [^{125}I]-(2.4G2 Fab) solutions that were diluted 10-fold with unlabelled 2.4G2 Fab (ratio = 0.9 ± 0.6). Greater than 98% of cell-bound [^{125}I]-(2.4G2 Fab) (2 nM, 30 min) was eluted by a low pH buffer, indicating that significant internalization did not occur.

3.2. *Monoclonal anti-DNP IgG1 binding to J774 cells*

Cell binding assays with 2 nM [^{125}I]-(2.4G2 Fab) in the presence of 30 μM DHK109.3 showed that apparent equilibrium was reached in 2–3 h. Subsequent competition assays were carried out with three hour incubation times. As shown in Fig. 2, the monoclonal anti-DNP IgG1 antibody ANO2 partially blocked [^{125}I]-(2.4G2 Fab) binding, but the blocking was not complete at the maximum antibody concentration (30 μM). In addition, as [^{125}I]-(2.4G2 Fab) binding was blocked, the cell supernatant radioactivity increased (Fig. 3). Similar results were also measured for the other four anti-DNP IgG1 monoclonal antibodies.

Curve-fitting analysis of the cell pellet radioactivities gave values for the IgG1–FcγRII association constant, K_m ranging from 8×10^4 M^{-1} (for DHK109.3) to 6×10^5 M^{-1} (for ANO5) (Table I). Analysis of the cell supernatant radioactivities gave consistent results.

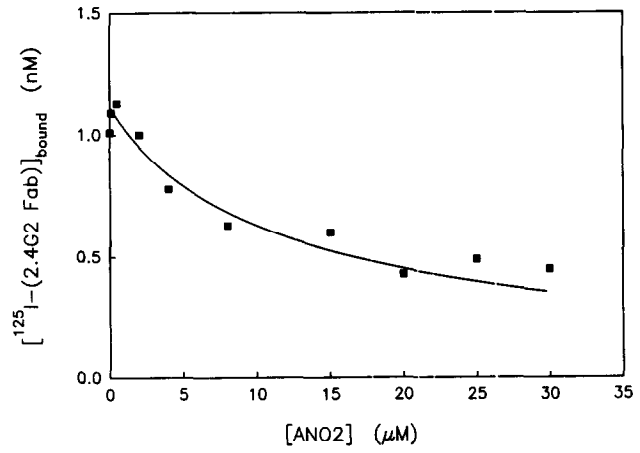

Fig. 2. Pellet data for IgG1 binding to J774 cells. The pellet radioactivities decreased when cells were treated for 3 h with 2 nM [^{125}I]-(2.4G2 Fab) and increasing amounts of ANO2. The association constant (K_m) for this curve is 4.9×10^5 M^{-1} with a Q of 3.2 nM.

3.3 *Effect of DNP-G on anti-DNP IgG1 association constants*

The association constants of DNP-G with three of the five monoclonal IgG1 antibodies were measured by tryptophan fluorescence quenching [18] and were as follows: 1B7.11, $(6.6 \pm 0.8) \times 10^6$ M^{-1}; ANO2, $(2.9 \pm 0.2) \times 10^6$ M^{-1}; DHK109.3, $(2.3 \pm 0.3) \times 10^5$ M^{-1}. The degree of fluorescence quenching for the other two antibodies (ANO5 and ANO6) was approximately equal to the amount of quenching measured for mouse IgG. However, these antibodies did bind to DNP (in that the antibodies were purified using DNP affinity chromatography). Therefore, the association constants for ANO5 and ANO6 were assumed to be about 10^4 M^{-1}. The IgG1-(DNP-G) association constants implied that > 90% of the hapten binding sites were saturated at the chosen DNP-G concentrations (115 μM for ANO2, 1B7.11 and DHK109.3; 850 μM for ANO5 and ANO6).

Analysis of cell pellet and supernatant data gave consistent K_m values. The values of K_m in the presence of DNP-G were not significantly different from the values in the absence of DNP-G, within experimental error (Table I).

Table I

Association constants K_m for anti-DNP IgG1 and FcγRII on J774 cells. Values for K_m were obtained by analyzing cell pellet or supernatant data using equations for reversible, bimolecular and competitive interactions between [^{125}I]-(2.4G2 Fab) and FcγRII and between mouse IgG1 and FcγRII [21]. Values are in units of 10^5 M^{-1}. Values in parentheses give the number of independently obtained binding curves.

Antibody	Cell Pellet DNP-G (−)	Supernatant DNP-G (−)	Cell Pellet DNP-G (+)	Supernatant DNP-G (+)	Average
ANO5	5.7 ± 4.5 (3)	4.2 ± 2.9 (3)	9.9 ± 3.0 (2)	6.0 ± 0.6 (2)	6.2 ± 2.1
ANO2	3.3 ± 0.6 (2)	8.2 ± 1.5 (2)	1.4 ± 0.7 (2)	1.4 ± 0.3 (2)	3.6 ± 3.0
ANO6	1.6 ± 0.5 (2)	3.0 ± 2.7 (2)	1.6 ± 0.4 (2)	3.0 ± 1.3 (2)	2.3 ± 0.7
1B7.11	1.3 ± 0.6 (3)	1.8 ± 1.0 (3)	2.3 ± 1.5 (3)	2.1 ± 1.6 (3)	1.9 ± 0.4
DHK109.3	0.9 ± 0.4 (3)	0.8 ± 0.5 (3)	0.7 ± 0.2 (2)	0.9 ± 0.3 (2)	0.8 ± 0.1

3.4. *Control Measurements for IgG1 binding to J774 cells*

The specificity of monoclonal IgG1 blocking of [^{125}I]-(2.4G2 Fab) was examined by treating cells with [^{125}I]-(2.4G2 Fab) (2 nM) and mouse polyclonal IgG F(ab)'$_2$ (2–26 μM). These measurements demonstrated that 26 μM mouse IgG F(ab)'$_2$ blocked less than 10% of the bound [^{125}I]-(2.4G2 Fab), indicating that the blocking data were Fc-specific. SDS-PAGE of overloaded gels visualized with silver staining showed that the 2.4G2 Fab preparation contained very little intact 2.4G2. In addition, when [^{125}I]-(2.4G2 Fab) was mixed with 2 mol% [^{125}I]-(2.4G2 IgG), DHK109.3 blocked the cell pellet radioactivity as effectively as with 100 mol% [^{125}I]-(2.4G2 Fab). Therefore, the incomplete blocking at long times and high IgG1 concentrations was not due to [^{125}I]-(2.4G2 IgG) in the [^{125}I]-(2.4G2 Fab) solutions.

Previous work has shown that monomeric, rabbit polyclonal IgG [8] and mouse myeloma IgG1, IgG2a and IgG2b [11] at 50 μM compete with and block > 90% of crosslinked, polyclonal rabbit IgG on P388D$_1$ cells. In this work, most of the monoclonal mouse IgG1 (at 30 μM) did not effectively block [^{125}I]-(2.4G2 Fab) binding. Thus, to examine whether the incomplete blocking was a property of the particular assay procedure used in this work, the ability of monomeric mouse IgG to block the binding of polyclonal mouse IgG that had been crosslinked with DSS and labelled with ^{125}I was

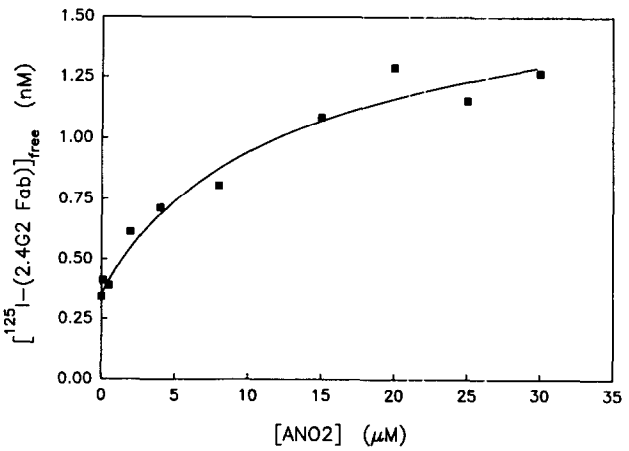

Fig. 3. Supernatant data for IgG1 binding to J774 cells. The supernatant radioactivities increased when cells were treated for 3 h with 2 nM [^{125}I]-(2.4G2 Fab) and increasing amounts of ANO2. The association constant (K_m) for this curve is 7.1×10^5 M^{-1}.

investigated. These measurements showed that [^{125}I]-(DSS-IgG) bound to J774 cells in a concentration-dependent and saturable manner, and that 30 μM polyclonal mouse IgG blocked about 80% of the bound [^{125}I]-(DSS-IgG), confirming the validity of the assay protocol. Thus, the observation that 30 μM anti-DNP IgG1 blocked only about 50% of the bound [^{125}I]-(2.4G2 Fab) was most likely a simple consequence of the high [^{125}I]-(2.4G2 Fab)–FcγRII association constant.

4. DISCUSSION

The measured values of K_m for the five monoclonal IgG1 antibodies with FcγRII on J774 cell surfaces (Table I) are consistent with previous measurements for mouse myeloma IgG1 on P388D$_1$ cells using competitive radioimmunoassays with ^{125}I-labelled, cross-linked polyclonal rabbit IgG (3×10^5 M^{-1}, 0°C) [11]. The K_m values are also consistent with previous immunofluorescence measurements for polyclonal IgG and monoclonal IgG1 on planar model membranes constructed from isolated J774 membrane fragments ($1–5 \times 10^5$ M^{-1}, 25°C) [18] or from purified and reconstituted FcγRII ($3–9 \times 10^5$ M^{-1}, 25°C) [20].

As shown in Table I, no large differences between different monoclonal IgG1 were detected within experimental error. The small differences that were observed in general arise from slightly altered Fc region conformations, flexibilities and/or solvent accessibilities. In addition, the binding of mouse macrophage (J774.1 and P388D$_1$) to planar lipid monolayers coated with monoclonal IgG1 antibodies was not strongly different for seven different antibodies, but one antibody (1B7) lacked the ability to promote macrophage-monolayer binding [25]. The 1B7 antibody is not equivalent to the 1B7.11 antibody. (M. Nakanishi, personal communications).

The observation that multivalent IgG complexes bind with higher affinity to macrophage cell surfaces than monomeric IgG [11] suggests that the key molecular event in the association of antibody-coated targets with macrophage cell surfaces (i.e. the onset of phagocytosis) is the multivalency of the target with respect to IgG Fc regions. However, in general, because previous experiments have been carried out primarily with crosslinked, multivalent IgG-antigen complexes [8,10], another possibility is that occupation of the antigen binding site enhances the IgG–FcγRII interaction. The association constants of anti-DNP IgG1 with FcγRII were not significantly affected by the presence of DNP-G at saturating concentrations, and the data therefore argue against a mechanism in which Fab occupation effects changes in Fc region function. A related set of measurements using fluorescence microscopy and FcγRII that was purified and reconstituted into substrate-supported planar membranes has given consistent results [26].

Acknowledgements: I like to thank Nancy Thompson for her support and advice during this project. Furthermore, I like to thank Helen Hsieh, Claudia Poglitsch, and Sheila Jones for assistance with biochemical preparations and helpful discussions. This work was supported by National Institutes of the Health Grant GM-37145 and a Department of Education Graduate Fellowship.

REFERENCES

[1] Metzger, H. (1978) Contemp. Top. Mol. Immunol. 7, 119–152.
[2] Brown, E.J. and Bekisz, J. (1984) J. Immunol. 132, 1346–1352.
[3] Colman, P.M. (1988) Adv. Immunol. 43, 99–132.
[4] Okada, A., Nakanishi, M., Tsurui, H., Wada, A., Terashima, M., and Osawa, T. (1985) Mol. Immunol. 22, 715–718.
[5] Burton, D.R. (1985) Mol. Immunol. 22, 161–206.
[6] Schlessinger, J., Steinberg, I.Z., Givol, D., Hochman, J. and Pecht, I. (1975) Proc. Natl. Acad. Sci. USA 72, 2775–2779.
[7] Mellman, I.S. and Unkeless, J.C. (1980) J. Exp. Med. 152, 1048–1069.
[8] Dower, S.K., DeLisi, C., Titus, J.A. and Segal, D.M. (1981) Biochemistry 20, 6326–6334.
[9] Qu, Z., Odin, J., Glass, J.D. and Unkeless, J.C. (1988) J. Exp. Med. 167, 1195–1210.
[10] Segal, D.M. and Hurwitz, E. (1977) J. Immunol. 118, 1338–1347.
[11] Segal, D.M. and Titus, J.A. (1978) J. Immunol. 120, 1395–1403.
[12] Ravetch, J.V. and Kinet, J.-P. (1991) Annu. Rev. Immunol. 9, 457–492.
[13] Unkeless, J.C. (1979) J. Exp. Med. 150, 580–596.
[14] Sumner, M.T. and Thompson, N.L. (1992) Biophys. J. 61, 1509.
[15] Balakrishnan, K., Hsu, F.J., Hafeman, D.G. and McConnell, H.M. (1982) Biochim. Biophys. Acta 721, 30–38.
[16] Leahy, D.J., Rule, G.S., Whittaker, M.M. and McConnell, H.M. (1988) Proc. Natl. Acad. Sci. USA 85, 3661–3665.
[17] Liu, F.-T., Bohn, J.W., Ferry, E.L., Yamamoto, H., Molinaro, C.A., Sherman, L.A., Klinman, N.R. and Katz, D.H. (1980) J. Immunol. 124, 2728–2737.
[18] Poglitsch, C.L. and Thompson, N.L. (1990) Biochemistry 29, 248–254.
[19] Pisarchick, M.L., Sumner, M.T. and Thompson, N.L. (1991) Biochemistry 30, 6662–6671.
[21] Sumner, M.T. (1992) Doctoral Dissertation, University of North Carolina at Chapel Hill.
[22] Mellman, I., Plutner, H. and Ukkonen, P. (1984) J. Cell Biol. 98, 1163–1169.
[23] Unkeless, J.C. and Healey, G.A. (1983) J. Immunol. Methods 56, 1–11.
[24] Daëron, M., Néauport-Sautès, C., Blank, U. and Fridman, W.H. (1986) Eur. J. Immunol. 16, 1545–1550.
[25] Kimura, K., Nakanishi, M., Ueda, M., Ueno, J., Nariuchi, H., Furukawa, S. and Yasuda, T. (1986) Immunol. 59, 235–238.
[26] Hsieh, H.V., Poglitsch, C.L. and Thompson, N.L. (1992) Biochemistry 31, 11562–11566.