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Analysis of PI (phosphatidylinositol)-anchoring antigens in a patient of paroxysmal nocturnal hemoglobinuria (PNH) reveals deficiency of 1F5 antigen (CD59), a new complement-regulatory factor

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FACS analysis together with PIPLC treatment was applied to PI-anchoring antigens such as DAF (decay-accerelating factor, CD55), 1F5 antigen (CD59), CD14 and CD16 on the cell surfaces of blood cells from a normal adult and a male patient with paroxysmal nocturnal hemoglubinuria (PNH). Through the extensive analysis, this patient proved to be completely defective in 1F5 antigen, a newly found complement-regulatory protein, on all the blood cells tested. In normal blood cells such as lymphocytes, monocytes and granulocytes, 1F5 antigen was expressed as one of PI-anchoring proteins. In contrast to most of PNH patients, this patient reserved DAF, CD14 and CD16 at normal levels in his erythrocytes, monocytes and granulocytes. Also, there were no significant differences between the normal adult and the patient in the activities of erythrocyte acetylcholinesterase and granulocyte alkaline phosphatase which were also known to be PI-anchoring enzymes. Thus, deficiency of 1F5 antigen must be deeply related to the clinical symptoms of PNH in this patient.

Phosphatidylinositol-anchoring protein; Paroxysmal nocturnal hemoglobinuria; Complement-regulatory protein

1. INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) has been known as an acquired clonal disorder of the bone-marrow stem cells that results in defects of the erythroid, monomyeloid and platelet cell-lineages. Recently, several authors [1-6] reported the expression of individual PI (phosphatidylinositol)-anchoring proteins to be markedly low on the surface of blood cells from the PNH patients. In this study, we estimated the expression of four PI-glycan-anchored antigens such as DAF [7,8], CD14 [9], CD16 [10,11] and 1F5 antigen [12] in granulocytes, lymphocytes, monocytes and erythrocytes from a male PNH patient, in comparison with those cells from a normal adult. DAF (decay accelerating factor, CD55) is a membrane protein of molecular mass 70 kDa with complement-regulatory activity, inhibiting the assembly of complement and accelerates the natural decay of the classical and alternative pathway C3 convertase. CD14 is a protein antigen on human monocytes as reported by Haziot et al. [9]. CD16, FcRIII receptor present in 135 000 sites on neutrophils, is characterized as one of the Fc receptors which bind IgG-containing immune complexes, leading to phagocytosis of the complex and activation of the neutrophil. 1F5 antigen (CD59) discovered by Okada et al. [12], is a membrane glycoprotein of 20 kDa which regulates the complement-activating reaction, i.e. hemolysis of neuraminidase-treated human erythrocytes via the alternative pathway. In spite of different sensitivity, these protein antigens have been shown to be released from blood cells by the action of PIPLC [7-12].

We also assayed the activity of acetylcholinesterase on red blood cells (RBC) and alkaline phosphatase on white blood cells (WBC). These enzymes were also reported to be PI-anchoring proteins [13-16].

2. MATERIALS AND METHODS

2.1. *Cell preparation*

Heparinated fresh blood was centrifuged at $700 \times g$ for 10 min. After removal of plasma and buffy coat, erythrocytes were washed three times with phosphate-buffered saline (PBS). White blood cells (lymphocytes, granulocytes and monocytes) were separated from fresh heparinated blood by centrifugation. The contaminating erythrocytes were removed by hypotonic lysis.

2.2. *PIPLC treatment*

Phosphatidylinositol-specific phospholipase C (PIPLC) was purified from the culture broth of *Bacillus thuringiensis* IAM 12077 according to the isolation procedures described by Ikezawa et al. [17]. Washed RBC (red blood cells) and WBC (white blood cells) were suspended in 0.25 M sucrose containing 10 mM Tris-HCl buffer (pH 7.4) at concentrations of 10^8 and 10^7 cells/ml, respectively. These cell suspensions (each 1 ml) were mixed with 1 U (for RBC) or 2 U (for WBC) of PIPLC and incubated for 20 min at 37°C. After incubation,

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the suspension was centrifuged at 700 × *g* 10 min. Precipitated cells were then washed two times with PBS, and subjected to FACS analysis. The resulting supernatants were then centrifuged at 40000 × *g* for 20 min, and subjected to enzyme analysis.

2.3. *FACS analysis*

PBS (25 μl) containing 10^6 cells were treated with 0.3–2.5 μg of corresponding antibodies in microtiter wells. Flow cytometric analysis was carried out with a Coulter Epics profile for 'single-color' analysis and a FACScan (Becton Dickinson) for 'two-color' analysis. In some groups, control cells were analyzed in the presence of rat or rabbit IgG immunoreagents for calculating the cytophilic binding.

2.4. *Enzyme assays*

The activities of PIPLC and alkaline phosphatase were determined according to the method described by Taguchi et al. [18]. The activity of acetylcholinesterase was determined by the method of Ellman et al. [19].

2.5. *Antibodies*

Anti-CD16 IgG(NKP-15) was purchased from Becton-Dickinson Co., and anti-CD14 IgG(MY4) and NKH-1 was from Coulter Co. Anti-DAF IgG was a gift from Dr T. Fujita. 1F5 IgG was kindly supplied by Dr N. Okada.

2.6. *Estimation of protein*

Protein contents were determined according to the method of Lowry et al. [20] with bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

3.1. *Expression of DAF on the blood cells from a normal adult and a PNH patient and sensitivity of DAF to PIPLC*

DAF on normal RBC, granulocytes and monocytes was expressed as a single population, but that on lymphocytes as multiple populations. By PIPLC treatment, DAF on RBC was reduced not more than 10%, as shown previously by Davitz et al. [7] and Medof et al. [8]. However, a significant amount of DAF was removed from the surfaces of granulocytes (32%), monocytes (48%) and lymphocytes (75%). Therefore, DAF generally exists as a PI-anchoring form in several blood cells from the normal adult, although it is not easily solubilized from RBC by PIPLC. Concerning to this point, Roberts et al. [21] reported that the resistance of acetylcholinesterase on human RBC to PIPLC treatment is due to the existence of an additional fatty acyl chain on the inositol ring which blocks the action of PIPLC.

DAF expression of this PNH patient was not essentially different from that of the normal adult on RBC,

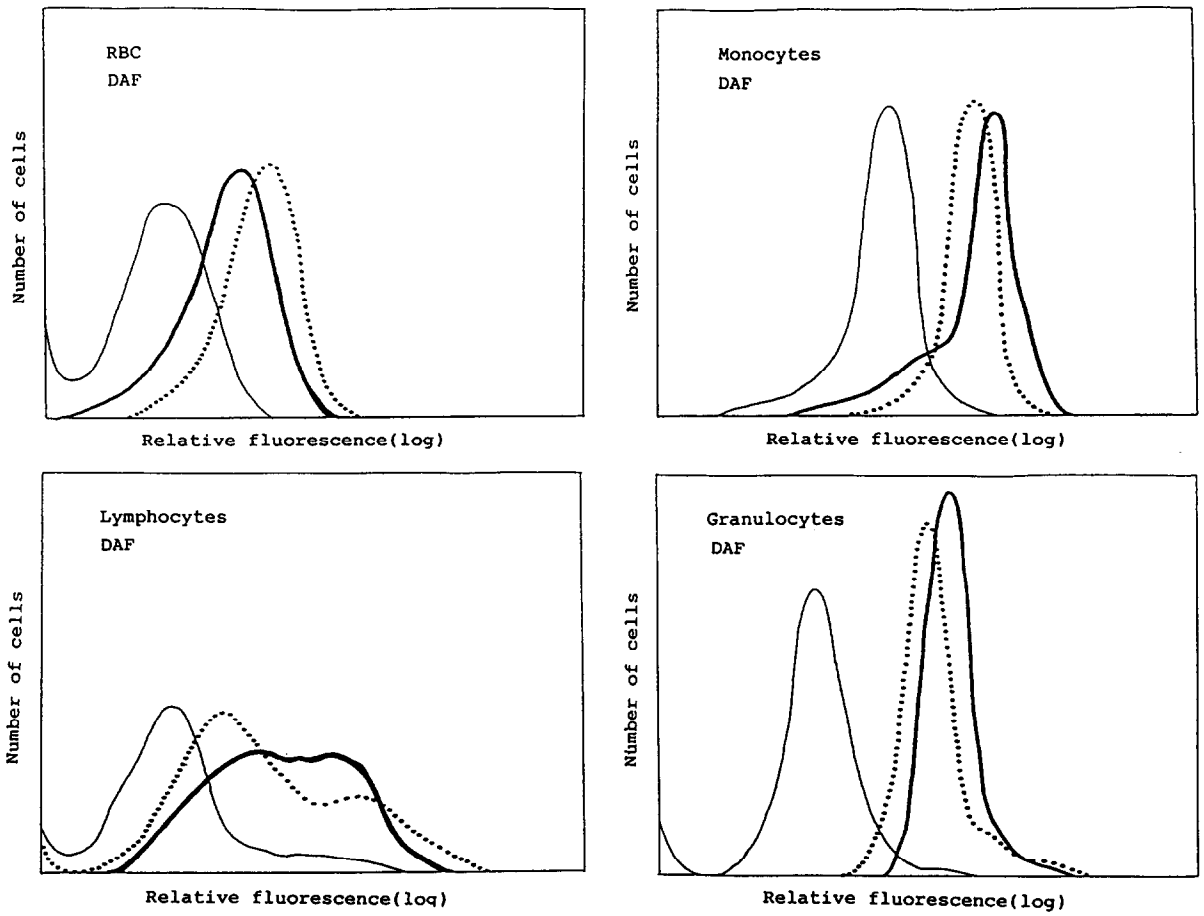

Fig.1. Comparative analysis of DAF expression on blood cells of the normal donor and a PNH patient. Cells were reacted with monoclonal mouse anti-DAF IgG(1), then stained with FITC-conjugated rabbit anti-mouse IgG. Control cells were directly stained with FITC-conjugated nonimmune mouse IgG(1) of FITC-conjugated rabbit anti-mouse IgG. (——), Control; (▬▬), the normal donor; (•••••), the PNH patient.

lymphocytes, monocytes and granulocytes (fig.1). Also in the patient cells, the extents of DAF solubilization with PIPLC is similar to those in normal cells (data not shown). According to Nicholson-Weller et al. [1,4], Kinoshita et al. [5], DAF deficiency was found in almost all cases of PNH patients they observed. In fact, DAF deficiency itself has been considered to be an essential index in the diagnosis of PNH. According to Ninomiya et al. [22], however, RBC from two of eight patients which they examined contained DAF at normal levels. Among the ten PNH patients which we are examaining and will report elsewhere, this patient is also unique in his full expression of DAF on RBC, as compared with other patients.

3.2. *Expression of 1F5 antigen and its sensitivity to PIPLC*

1F5 antigen on normal blood cells was expressed as a single population in RBC, monocytes and granulocytes, except in lymphocytes (fig.2). By PIPLC treatment, the content of 1F5 antigen was significantly decreased in granulocytes (55%), monocytes (54%) and lymphocytes (80%). Like DAF, 1F5 antigen were not released from RBC by PIPLC not more than 15%, although this antigen was demonstrated by Okada et al. [12] to be anchored with PI to RBC membrane. On the other hand, 1F5 antigen was completely defective in all kind of patient blood cells tested, as shown in fig.2. Therefore, clinical symptoms of PNH in this patient, such as sucrose hemolysis and complement activated hemolysis in Ham test, must be related to 1F5 antigen deficiency.

Complement-regulatory protein antigens similar to DAF and 1F5 antigen have been shown as membrane cofactor protein [23], homologous restriction factor (HRF) [24], C8-binding protein [25] and P-18 protein [26]. Among these proteins, P-18 protein seems to be immunologically identical to 1F5 antigen (personal communication).

3.3. *Expression of CD14 and CD16 antigens and their sensitivity to PIPLC*

Fig.3 shows expression of CD16 in granulocytes and lymphocytes, and CD14 in monocytes from a normal adult and the PNH patient, and sensitivity of these an-

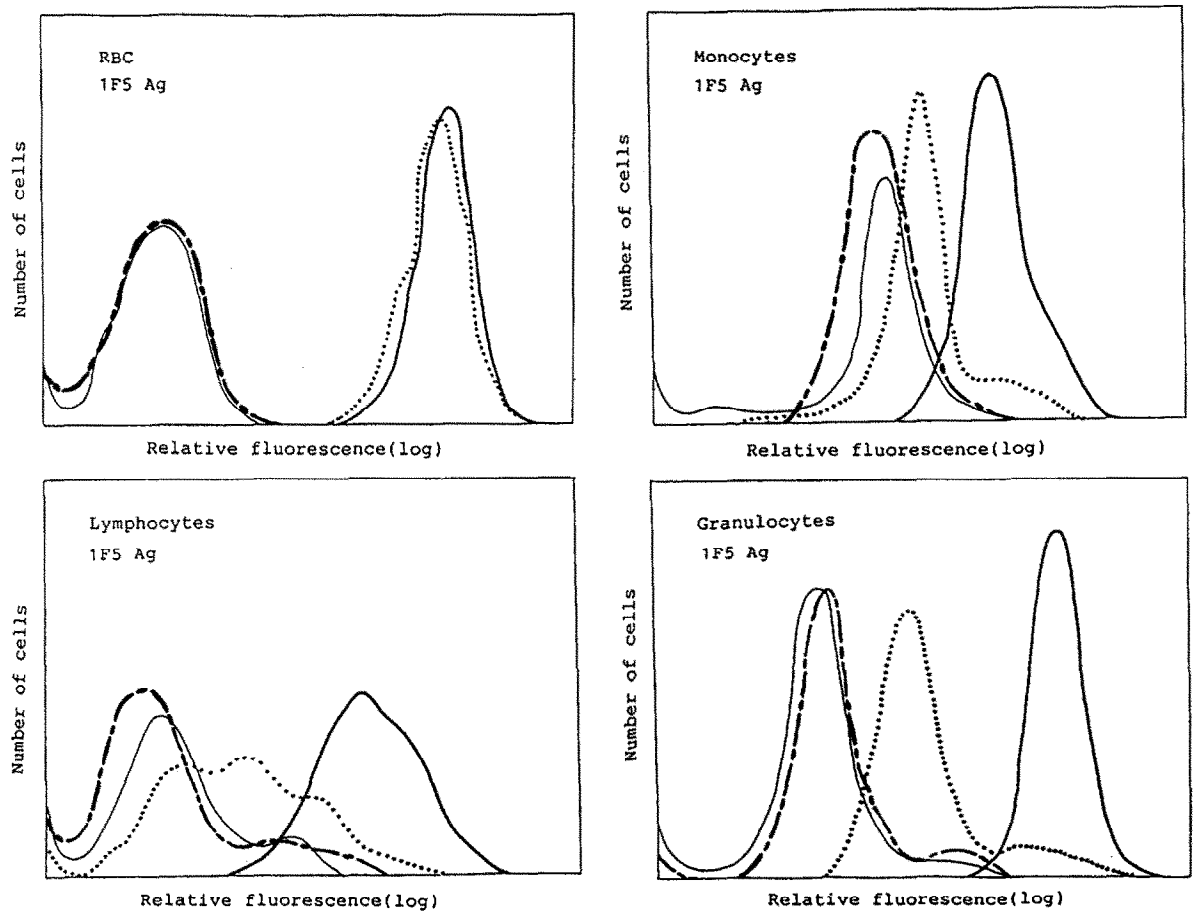

Fig.2. Comparative analysis of 1F5 antigen expression on blood cells of the normal donor and the PNH patient. PIPLC-treated and -untreated cells were reacted with monoclonal mouse 1F5 IgG(1), then stained with FITC-conjugated rabbit anti-mouse IgG. Control cells were treated in the same way as in fig.1. (——), Control; (▬▬), the normal donor (PIPLC-untreated); (•••••), the normal donor (PIPLC-treated); (- - - -), the PNH patient (PIPLC-untreated).

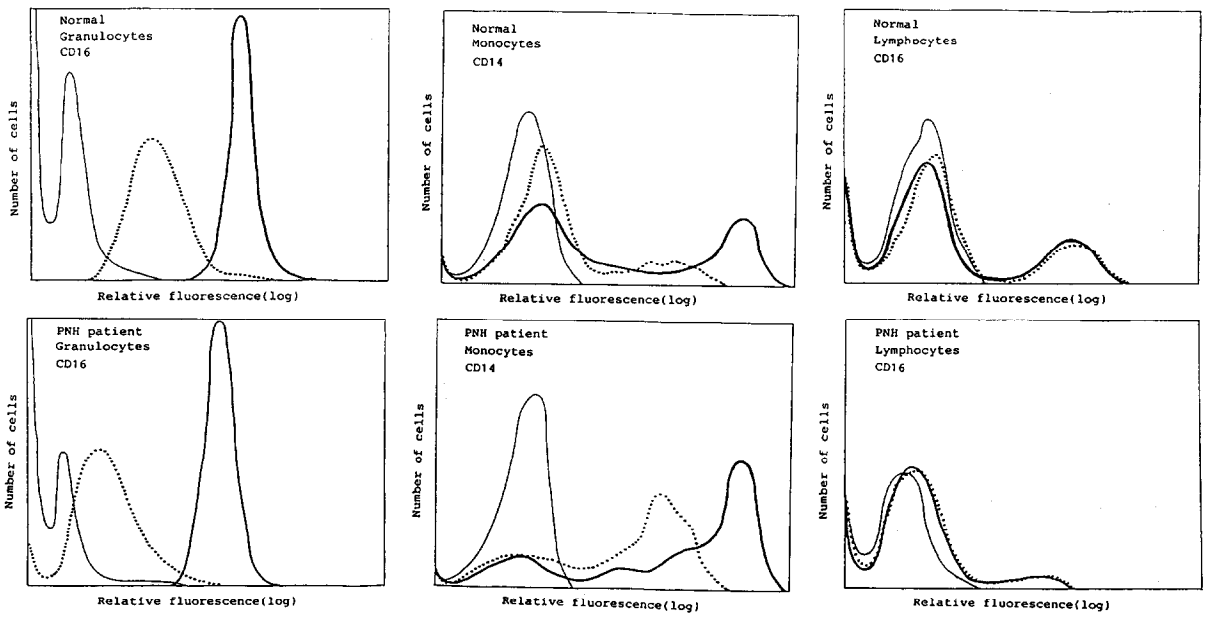

Fig.3. FACS analysis of CD16 and CD14 expression on blood cells of the normal donor and the PNH patient. PIPLC-treated and -untreated cells were stained with FITC-conjugated mouse anti-CD16 IgG(1) or FITC-conjugated mouse anti-CD14 IgG(2). Control cells were directly reacted with FITC-conjugated mouse nonimmune IgG(1) or FITC-conjugated mouse nonimmune IgG(2b). (——), Control; (▬▬), PIPLC-untreated; (•••••), PIPLC-treated.

tigens to PIPLC. CD14 was exclusively expressed on monocytes from the patient as well as the normal adult. Similarly, CD16 was mainly expressed on granulocytes from these two persons. CD14 and CD16 were effectively released by PIPLC from monocytes (20%) and granulocytes (50%), respectively. In the present study, however, an interesting subpopulation containing PIPLC-insensitive CD16 was found in normal and PNH lymphocytes. This CD16 in PNH lymphocytes was expressed at very low level. This subpopulation proved to be the population of NK cells, since it was double-stained with monoclonal antibody NKH-1 and anti-CD16 IgG. However, our recent study (will be published later) suggests that low expression of CD16 on NK cells observed in this patient is not a common feature of PNH patients; only two out of ten PNH patients show low expression of CD16 on NK cells. Recently, Lanier et al. [27] reported that CD16 was expressed on NK cells as well as leukocytes, and that molecular size of CD16 on NK cells were slightly different from that on leukocytes. These results suggest the possibility that CD16 on NK cells is not to be a PI-anchoring protein, but to be anchored to the membrane by a C-terminal, hydrophobic peptide tail.

3.4. *Expression of PI-anchoring enzymes in blood cells*

There were no significant differences between the normal adult and the patient in the activity levels of acetylcholinesterase on RBC ghosts and of alkaline phosphatase on white blood cells (mainly on granulocytes).

Recently, growing interest has been directed to physiological significance of PI-anchoring proteins. For instance, expression of these proteins on blood cells seems to be directly related to the cell differentiation. In fact, decrease or deficiency in PI-anchoring proteins in PNH patients suggests that PNH is caused by clonal disorder or irregular differentiation in these cells.

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Appendix: During preparation of this paper Ravetech and Perussia [28] have reported that CD16 observed in NK cell is resistant to PIPLC treatment, encoded in a different gene from that of granulocyte and expressed as a non PI-anchoring form.

REFERENCES

[1] Nicholson-Weller, A., March, J.P., Rosenfeld, S.I. and Austen, K.F. (1983) Proc. Natl. Acad. Sci. USA 80, 5066–5070.
[2] Pangburn, M.K., Schreiber, R.D. and Müller-Eberhard, H.J. (1983) Proc. Natl. Acad. Sci. USA 80, 5430–5434.
[3] Rotoli, B., Robledo, R., Scarpato, N. and Luzzatto, L. (1984) Blood 64, 847–851.
[4] Nicholson-Weller, A., Spicer, D.B. and Austen, K.F. (1985) N. Eng. J. Med. 312, 1091–1907.
[5] Kinoshita, T., Medof, M.E., Silber, R. and Nussenzweig, V. (1985) J. Exp. Med. 162, 75–92.

[6] Mansch, G.M., Weller, P.F. and Nicholson-Weller, A. (1988) Blood 72, 1089–1092.
[7] Davitz, M.A., Low, M.G. and Nussenzweig, V. (1986) J. Exp. Med. 163, 1150–1161.
[8] Medof, M.E., Walter, E.L., Roberts, W.L., Haas, R. and Rosenberry, T.L. (1986) Biochemistry 25, 6740–6747.
[9] Haziot, A., Chen, S., Ferrero, E., Low, M.G., Silber, R. and Goyert, S.M. (1988) J. Biol. Chem. 141, 547–552.
[10] Selvaraj, P., Rosse, W.F., Silber, R. and Springer, T.A. (1988) Nature 339, 565–567.
[11] Huizinga, T.W.J., Van der Schoot, C.E., Jost, C., Klassen, R., Kleijer, M., Von der Borne, A.E.G., Roos, D. and Tettetoo, P.A.T. (1988) Nature 333, 667–669.
[12] Okada, N., Harada, R., Fujita, T. and Okada, H. (1989) Int. Immunol. 1, 205–208.
[13] Ikezawa, H., Yamanegi, M., Taguchi, R., Miyashita, T. and Ohyabu, T. (1976) Biochim. Biophys. Acta 450, 154–164.
[14] Taguchi, R. and Ikezawa, H. (1978) Arch. Biochem. Biophys. 186, 196–201.
[15] Low, M.G. and Finean, J.B. (1977) FEBS Lett. 82, 143–146.
[15] Ikezawa, H. and Taguchi, R. (1981) Methods Enzymol. 71, 731–741.
[17] Ikezawa, H., Nakabayashi, T., Suzuki, K., Nakajima, M., Taguchi, T. and Taguchi, R. (1983) J. Biochem. 93, 1717–1719.
[18] Taguchi, R., Asahi, Y. and Ikezawa, H. (1980) Biochim. Biophys. Acta 619, 48–57.
[19] Ellman, G.L., Courtney, K.D., Andres, V. jr and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95.
[20] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[21] Roberts, W.L., Myher, J.J., Kuksis, A., Low, M.G. and Rosenberry, T.L. (1988) J. Biol. Chem. 263, 18766–18775.
[22] Ninomiya, H., Abe, T., Shichishima, T., Terasawa, T. and Fujita, T. (1988) Br. J. Haematol. 69, 81–87.
[23] Cole, J.L., Houstley, G.A. jr, Dykman, T.R., MacDermott, R.P and Atkinson, J.P. (1985) Proc. Natl. Acad. Sci. USA 82, 859–863.
[24] Zalman, L.S., Wood, L.M., Franke, M.K. and Müller-Eberhard, H.J. (1986) Proc. Natl. Acad. Sci. USA 83, 6975–6979.
[25] Schonenermark, S., Rauterberg, E.W., Shin, M.L., Loke, S., Roelcke, D. and Hansch, G.M. (1986) J. Immunol. 136, 1772.
[26] Sugita, Y., Nakano, Y. and Tomita, M. (1988) J. Biochem. 104, 633–637.
[27] Lanier, L.L., Ruitenberg, J.J. and Philips, J.H. (1988) J. Biol. Chem. 141, 3478–3485.
[28] Ravetech, J.V. and Perussia, B (1989) J. Exp. Med. 170, 481–497.