Estimation of B-Cells Transformed by Epstein-Barr Virus in Patients with Congenital Agammaglobulinemia

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TSUCHIYA, S., NAKAE, S., KONNO, T., TADA, K. and ONO, Y. *Estimation of B-Cells Transformed by Epstein-Barr Virus in Patients with Congenital Agammaglobulinemia.* Tohoku J. exp. Med., 1981, 135 (4), 379-385 — In vitro immunoglobulin synthesis was measured in lymphocytes from four patients with congenital agammaglobulinemia (cA γ) stimulated by two different polyclonal B-cell activators, pokeweed mitogen (PWM) and Epstein-Barr virus (EBV). In PWM-stimulated cultures, patient T-cells treated with mitomycin C (MMC) were able to help the immunoglobulin (Ig) synthesis of normal B-cells. Patient B-cell-enriched fraction not containing surface Ig positive cells did not produce Ig in combination with MMC-treated autologous or allogeneic T-cells. Patient lymphocytes were infected with EBV and the subsequent production of Ig was measured. In lymphocytes from control subjects, exponential growth of the cells having EBV-associated nuclear antigen (EBNA) was shown to be associated with an exponential increase in Ig secretion within 1 week after EBV infection. However, in lymphocytes from three of the four patients, it took 2, 4 and 10 weeks, respectively, until lymphocyte-transformation and subsequent Ig-secretion were observed. Lymphocytes from one patient were not transformed nor did they secrete Ig after EBV infection. These results may imply that a small number of B-cells are present in peripheral blood of most of patients with cA γ , and that they are able to produce Ig after transformation by EBV which takes a much longer time than in controls. ——— B cell; Epstein-Barr virus; congenital agammaglobulinemia

Patients with congenital agammaglobulinemia (cA γ) are considered to lack or have few B-cells as defined by surface Ig (sIg) Epstein-Barr virus, (EBV) receptors, and anti-B-lymphocyte-sera positive cells (Hayward and Greaves 1975; Siegal et al. 1971). However, we have reported recently that peripheral lymphocytes of two of three patients with cA γ who lacked B-lymphocytes with sIg were transformed by EBV, giving rise to lymphoblastoid cell lines (LCL) (Tsuchiya et al. 1980). These data suggest the presence of B-lymphocytes with EBV receptors in the peripheral blood of those patients with cA γ .

In view of such evidence we tried to examine the possibility of lymphocyte transformation by EBV infection and subsequent Ig production in order to estimate roughly the number of B-cells in patients with cA γ .

MATERIALS AND METHODS

Patients. Peripheral blood lymphocytes were obtained from four patients with a primary diagnosis of cA γ . They were previously described in detail (Tsuchiya et al. 1980), except for Patient S.T. All patients lacked B-cells in their peripheral blood in terms of sIg positive lymphocytes and had rosette-forming-cells with sheep erythrocytes (SRBC) at a level of more than 90 percent. The concentration of serum IgM was 40 mg/100 ml, IgG 165 mg/100 ml, and IgA 30 mg/100 ml. They received periodical replacement therapy with γ -globulin preparations.

Lymphocyte preparation. Mononuclear cells were isolated from the peripheral blood of patients and control subjects by Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) gradient centrifugation (Böyum 1968). T-enriched or B-enriched fractions were obtained from the mononuclear cells by density sedimentation of spontaneous SRBC-rosettes using SRBC pretreated with neuraminidase (Behringwerke, Marburg, W. Germany) (Siegal et al. 1978; Weiner et al. 1973). Nonrosetting mononuclear cells, termed B-enriched fraction, contained more than 60 percent sIg positive cells in control subjects. The pellet of rosetting mononuclear cells, the T-enriched fraction, was treated with Tris-ammonium chloride buffer (pH 7.65) to remove SRBC. It contained more than 90 percent spontaneous rosette forming cells with SRBC.

PWM stimulated lymphocyte culture. All lymphocytes were cultured in RPMI 1640 medium supplemented with kanamycin (60 μ g/ml) and 20% heat-inactivated fetal calf serum. B-enriched fractions (1×10^5 /ml) from patients (Bp) and control subjects (Bn) were mixed with 4×10^5 /ml autologous and allogeneic T-enriched fractions (Tp and Tn) in 1 ml culture medium, and cultured with pokeweed mitogen (PWM) (GIBCO, Grand Island, New York) at a dilution of 1/100 (v/v) at 37°C with 5% CO $_2$ in air for 7 days. In some cultures, Tp and Tn were treated with 25 μ g/ml of mitomycin C (MMC) at 37°C for 30 min in order to eliminate suppressor activity of those fractions (Lipsky et al. 1978). After 7 days' incubation, the production of immunoglobulin secreted in culture supernatants was measured by inhibition radioimmunoassay.

Transformation of lymphocytes by infection with EBV. Peripheral mononuclear cells were infected with EBV and cultured in a microculture plate according to a previously described method (Tsuchiya et al. 1980). Briefly, pellets of 3×10^6 mononuclear cells were infected with EBV preparation from the B95-8 marmoset cell line at an average multiplicity of infection (MOI) of 0.17. After 60 min at 37°C, the cells were spun down and adjusted to 3×10^6 /ml in culture medium and then distributed into Microtest II plates (No. 3042, Falcon, Oxnard, Calif.) at a concentration of 6×10^5 /0.2 ml/well and cultured at 37°C in an atmosphere of 5% CO $_2$ in air. One tenth ml of culture supernatant was collected from each well once a week for measurement of secreted Ig and 0.1 ml of fresh culture medium was added to each well. The microcultures were continued until the wells were fully occupied by the transformed cells.

Detection of EBV-associated nuclear antigen (EBNA) was carried out according to the method of Reedman and Klein (1973).

Measurement of Ig. IgG and IgM secreted in culture supernatants were determined by inhibition radioimmunoassay as described by Platts-Mills and Ishizaka (1975). IgG and IgM purified from normal human sera labeled with 125 I (The Radiochemical Center, Amersham). One tenth ml of diluted culture supernatants or standard immunoglobulin solutions (5–1000 ng/ml) were mixed with 0.1 ml of rabbit anti-human IgG or rabbit anti-human IgM serum diluted in phosphate-buffered saline (PBS) containing 1% normal rabbit serum and 2% fetal calf serum. After 120-min incubation at room temperature, 0.1 ml of 125 I-labeled IgG or IgM (50 ng/ml) was added and 120 min later 0.1 ml of sheep anti-rabbit IgG serum was added. The mixture was allowed to stand overnight at 4°C. The precipitate was washed three times with cold PBS and its radioactivity was determined by a well type gamma counter (Aloka, Tokyo). This assay allowed for detection of 10–500 ng/ml of IgG and IgM of culture supernatants.

RESULTS

In vitro Ig synthesis induced by PWM. A mixture of autologous and allogeneic B- and T-cells from patients and controls was cultured in vitro with PWM. PWM-induced Ig syntheses in those cultures are shown in Fig. 1. Normal B-cells produced IgM and IgG in culture with normal T-cells. Cultured with normal T-cells treated with MMC (TnM), normal B-cells showed a 2- to 20-fold increase in IgG and IgM secretion. While MMC-untreated patient T-cells failed to induce Ig production in normal B-cells, MMC-treated patient T-cells were able to induce Ig production in normal B-cells. On the other hand, patient B-cells which were obtained from the nonrosetting mononuclear cell fraction did not produce Ig in any mixtures of patient and normal T-cells either untreated or treated with MMC.

Fig. 1. Immunoglobulin production of the B-cell-enriched fraction from patients with congenital agammaglobulinemia. Autologous and allogeneic T-B combinations were cultured in the presence of PWM for 7 days. Subsequently, culture supernatants were harvested, and IgM (open columns) and IgG (dotted columns) secretions were quantified by inhibition radioimmunoassay. Bp and Tp, B-cell- and T-cell-enriched fraction from patients; Bn and Tn, B-cell- and T-cell-enriched fraction from control subjects; TpM and TnM, T-cell-enriched fraction treated with mitomycin C.

Correlation between Ig production and EBNA positive cells. In order to know the correlation between the increase in Ig secretion in culture supernatants and the growth of cells transformed by EBV, we determined the number of EBNA positive cells and the concentration of Ig secreted into the medium at various times after infection with EBV by using lymphocytes from a control subject. As shown in Fig. 2, it was noticed that IgM and IgG secretion of lymphocytes infected with EBV increased in accordance with the increase in number of EBNA positive cells.

Fig. 2. Immunoglobulin production by EBNA positive cells. Lymphocytes from a control subject were infected with EBV and cultured in Molton tubes at a concentration of $1 \times 10^6/\text{ml}$. Culture supernatants were harvested every day for seven days after counting the viable cell number. The absolute number of EBNA-positive cells was determined from the cell count for each tube and the percent of EBNA-positive cells. IgM (○—○) and IgG (●—●) secretions were quantified by inhibition radioimmunoassay.

Ig secretion by EBV-induced LCL. Ig secretion by EBV-induced LCL from patients was examined and compared with that from control subjects (Fig. 3). In these experiments LCL from patients secreted only IgM class of Ig. When lymphocytes from the four control subjects were infected with EBV, approximately $10 \mu\text{g}/\text{ml}$ of IgM were secreted in culture supernatants within a week after EBV infection. On the other hand, the lymphocytes from patients secreted only a negligible amount of IgM ($<1 \mu\text{g}/\text{ml}$) a week after EBV infection. However, a logarithmic increase in Ig production was observed during the 2nd week of infection with EBV in Patient S.T., the 4th week of infection in Patient M.O. and the 10th

Fig. 3. Time course of immunoglobulin secretion by EBV-infected lymphocytes from control subjects (a) and patients with congenital agammaglobulinemia (b). EBV-infected lymphocytes were distributed into Microtest II plate (No. 3042, Falcon) and cultured at 37°C in 5% CO₂ in air. One tenth ml of culture supernatant was harvested from each well once a week and pooled for measurement of secreted Ig by inhibition radio-immunoassay.

week of infection in Patient O.S. Cell clumps and acid production were observed before the increase in Ig secretion (Miller and Lipman 1973). Lymphocytes from one patient (K. Ko.) secreted a small amount of Ig (less than 1 µg/ml) sometime after infection with EBV but did not give rise to LCL.

DISCUSSION

Patients with cA γ have been said to have no detectable B-cells or EBV receptor positive cells in their peripheral blood as measured by membrane immunofluorescence or EBV adsorption techniques (Hayward and Greaves 1975; Gupta and Good 1977). However, we previously reported that lymphocytes from two of three patients with cA γ were transformed by EBV, giving rise to LCL (Tsuchiya et al. 1980). In this study, we have confirmed our previous report and observed a lapse of transformation after EBV infection of patient lymphocytes and a subsequent increase in Ig production. There was a strong correlation between the amount of secreted Ig and the increase in cell number of EBNA positive cells during the early period in transformed lymphocytes of controls.

Two weeks elapsed in Patient S.T., 4 weeks in Patient M.O. and 10 weeks in Patient O.S. until sufficient amounts of secreted Ig (>10 µg/culture) were detected in culture supernatants. As previously mentioned, lymphocytes from

Patient K. Ko. were not transformed at all (Tsuchiya et al. 1980). Such different time lapses before distinct increases in secretion of Ig among the patients might indicate the presence of different amounts of target cells for EBV in these patients. In view of the fact that sIg-bearing cells are the target of transformation by EBV (Katsuki et al. 1977), the presence of a small amount of B-cells in the peripheral blood might be considered in Patients S.T., M.O. and O.S. In K. Ko., B-lymphocytes might be absent.

In PWM-stimulated cultures, the presence of suppressor T-cells has been reported by others (Schwarz et al. 1977; Lipsky et al. 1978; Siegal et al. 1978). When treated with MMC, these T-cells are able to help normal B-cells differentiate into Ig producing cells. These characteristics of T-cells from patients with cA γ resemble those of T-cells from cord blood (Tosato et al. 1980), and do not seem to be a unique feature of cA γ . The most prominent finding for PWM-stimulated cultures is the lack of a sufficient amount of patient B-cells able to differentiate into Ig secreting cells.

While B-cells from four patients, in terms of non-E-rosetting lymphocytes, have no detectable amount of Ig in culture supernatants in combination with MMC-treated T-cells, in three patients the LCL established by infection with EBV produced Ig which was secreted into culture supernatants suggesting the presence of B-cells, even though the number was extremely small.

Acknowledgments

This study was supported in part by a Research Grant from the Intractable Disease Division, Public Health Bureau, Ministry of Health and Welfare, Japan.

References

- 1) Böyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. clin. Lab. Invest.*, Suppl. 97, 1-76.
- 2) Gupta, S. & Good, R.A. (1977) Subpopulation of human T lymphocytes. I. Studies in immunodeficient patients. *Clin. exp. Immunol.*, 30, 222-228.
- 3) Hayward, A.R. & Greaves, M.F. (1975) Central failure of B-lymphocyte induction in pan-hypogammaglobulinemia. *Clin. Immunol. Immunopathol.*, 3, 461-470.
- 4) Katsuki, T., Hinuma, Y., Yamamoto, N., Abo, T. & Kumagai, K. (1977) Identification of the target cells in human B lymphocytes for transformation by Epstein-Barr virus. *Virology*, 83, 287-294.
- 5) Lipsky, P.E., Ginsburt, W.W., Finkelman, F.D. & Ziff, M. (1978) Control of human B lymphocyte responsiveness: Enhanced suppressor T cell activity after in vitro incubation. *J. Immunol.*, 120, 902-910.
- 6) Miller, G. & Limpan, M. (1973) Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. *Proc. nat. Acad. Sci. USA*, 70, 190-194.
- 7) Platts-Mills, T.A.E. & Ishizaka, K. (1975) IgG and IgA diphtheria antitoxin responses from human tonsil lymphocytes. *J. Immunol.*, 114, 1058-1064.
- 8) Reedman, B.M. & Klein, G. (1973) Cellular localization of an Epstein-Barr virus (EBV)-associated complement-fixing antigen in producer and nonproducer lymphoblastoid cell lines. *Int. J. Cancer*, 11, 499-520.
- 9) Schwartz, S.A., Choi, Y.S., Shou, L. & Good, R.A. (1977) Modulatory effects on immunoglobulin synthesis and secretion by lymphocytes from immunodeficient patients. *J. clin. Invest.*, 59, 1176-1187.

- 10) Siegal, F.P., Pernis, B. & Kunkel, H.G. (1971) Lymphocytes in human immunodeficiency states: A study of membrane-associated immunoglobulins. *Europ. J. Immunol.*, **1**, 482-486.
 - 11) Siegal, F.P., Siegal, M. & Good, R.A. (1978) Role of helper, suppressor and B-cell defects in the pathogenesis of the hypogammaglobulinemias. *New Engl. J. Med.*, **299**, 172-178.
 - 12) Tosato, G., Magrath, I.T., Koshi, I.R., Dooley, N.J. & Blaese, R.M. (1980) B cell differentiation and immunoregulatory T cell function in human cord blood lymphocytes. *J. clin. Invest.*, **66**, 383-388.
 - 13) Tsuchiya, S., Konno, T., Tada, K. & Ono, Y. (1980) Epstein-Barr virus-induced lymphoblastoid cell lines from patients with primary immunodeficiency diseases. *Scand. J. Immunol.*, **11**, 155-162.
 - 14) Weiner, M.S., Bianco, C. & Nussenzweig, V. (1973) Enhanced binding of neuraminidase-treated sheep erythrocytes to human T lymphocytes. *Blood*, **42**, 939-946.
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