Immunological Abnormality of Peripheral Blood B Cells in Patients with Autoimmune Thyroid Disease

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Abstract

We investigated the response to immunoglobulin G-secreting cells (ISC) by peripheral blood mononuclear cells (PB-MNC) and purified B cells following stimulation with Staphylococcus aureus Cowan 1 (SAC) or with B cell stimulatory factor 2 (interleukin 6: IL-6), using the reverse hemolytic plaque assay in an attempt to clarify the immunological functions of peripheral blood B cells in patients with autoimmune thyroid disease (AITD). ISC response by PB-MNC following stimulation with SAC was significantly decreased in patients in the hyperthyroid state of Graves' disease and Hashimoto's thyroiditis as compared with that of normal controls. The difference in SAC-response was not significant between patients with euthyroid state of Graves' disease and normal controls. ISC response by PB-MNC following stimulation with SAC exhibited a reciprocal relationship to TRAb in patients with Graves’ disease. Using purified B cells, some spontaneous ISC response without SAC stimulation was observed in patients in the hyperthyroid state of Graves' disease and Hashimoto's thyroiditis. This spontaneous ISC response was further enhanced by IL-6. These results suggest that in organ-specific autoimmune diseases such as AITD, immunological abnormalities exist in B cells and some B cells are nonspecifically activated in the immunologically active state.

Autoimmune thyroid disease (AITD) is characterized by aberrant functions of immunocompetent cells, and high titers of various kinds of thyroid-specific autoantibodies. Concerning AITD, there have been conflicting opinions about the defect of immunoregulation, namely whether the defect is a nonspecific generalized defect or a specific single defect. Up to date, these discrepancies have been mainly observed in investigations of the suppressor T cell defect (Volpé, 1985). However, some groups of investigators suggested that the primary defect of immunoregulation in AITD resides in the B cell function (Beall, 1982; De Bernardo and Davies, 1983; Jones et
al., 1982).

With regard to B cell function in AITD, recently Weetman et al. reported that autoantigen-specific B cells previously activated exist in peripheral blood. However, these autoantigen-specific B cells are found in several different functional stages. Thus, these circulating B cells from such patients do not manifest a uniform response to B cell stimulators. Furthermore, they showed that these B cells responded normally to nonspecific stimulators (Weetman et al., 1985). On the other hand, De Bernardo and Davies indicated that nonspecific abnormalities of B cell function are also present in AITD (De Bernardo and Davies, 1983). The maturation of B cells from their resting stage to an immunoglobulin (Ig)-secreting plasma cell involves discrete steps of activation, proliferation and differentiation (Howard and Paul, 1983; Muraguchi et al., 1983; Yoshizaki et al., 1982). At each step, specific signals are necessary to direct the B cells. B cells can be activated by antigens or surface Ig cross-linking agents such as anti-Ig antibody or Staphylococcus aureus Cowan 1 (SAC) which is known to activate and proliferate resting B cells and then differentiate into Ig-secreting cells by T cell help (Falkoff et al., 1982). In the present study, we examined the immunoglobulin G-secreting cell (ISC) response by peripheral blood mononuclear cells (PB-MNC) following stimulation with SAC or by B cells following stimulation with interleukin 6 (IL-6) to clarify the immunological functions of circulating B cells in AITD.

Materials and Methods

Subjects

The subjects in this investigation include 29 patients with Graves' disease, 10 patients with Hashimoto's thyroiditis and 14 normal controls. The diagnosis of AITD was confirmed by clinical, biochemical and hormonal features. The patients with Graves' disease consisted of 14 untreated patients and 15 patients in the euthyroid state under a regimen of methimazole.

Preparation of PB-MNC and B cells

PB-MNC were isolated from heparinized peripheral blood from healthy controls or AITD patients using lymphocyte separation medium (Litton Bionetics, Kensington, MD). The recovered cells were washed with 10mM phosphate-buffered saline (PBS), pH 7.2, and were resuspended at a concentration of $1 \times 10^6$/ml in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) containing 10% fetal calf serum (FCS; Grand Island Biological Co., Grand Island, NY), 50 U/ml penicillin, and 50 µg/ml streptomycin (Flow Laboratories, North Ryde, N.S.W., Australia). This preparation was used as PB-MNC. To prepare B cells, the PB-MNC was incubated in plastic culture dishes (Falcon 3002, Becton-Dickinson, Oxnard, CA) at 37°C for 2h in 5% CO2 and 95% air. After incubation, nonadherent cells were removed by three gentle washes with PBS. The recovered cells were incubated with sheep erythrocytes in RPMI 1640 medium at 4°C for 2h, and rosette-forming cells with sheep erythrocytes were removed by centrifugation over lymphocyte separation medium. This procedure was repeated twice. Non-rosette-forming cells were then treated with 100 µl of $10^{-1}$ diluted anti-monocyte monoclonal antibody (OKM 1; Ortho Diagnostic Systems, Westwood, MA), 100 µl of $10^{-1}$ diluted anti-T cell monoclonal antibody (OKT 3; Ortho Diagnostic Systems) and 200 µl of $5 \times 10^{-2}$ diluted rabbit complement (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) at 37°C for 1h, and washed with PBS. The recovered cells were used as purified B cells. This cell population contained more than 95% surface Ig-positive cells, less than 1% OKM 1-positive cells, and less than 1% OKT 3-positive cells as detected by fluoresceinated monoclonal antibody.

Assay of ISC production from PB-MNC or B cells

PB-MNC ($1 \times 10^6$) or purified B cells ($5 \times 10^4$) were cultured in 0.2 ml RPMI medium containing 10% FCS in round bottomed microtiter culture plates (Nunc) in the presence or absence of SAC (0.01%; Behring Diagnostics, La Jolla, CA) or human recombinant IL-6 (5 U/ml; this
concentration was confirmed to be optimal for ISC generation by basic studies) at 37°C in 5% CO₂ and 95% air. IL-6 was donated by Drs. T. Hirano and T. Kishimoto, Osaka University, Osaka, Japan (Hirano et al., 1986; Hirano et al., 1987). The detection of ISC was performed on day 5 by a modified Jerne plaque-forming cells (PFC) assay on slide glasses using protein A (Pharmacia Fine Chemicals, Uppsala, Sweden)-coated sheep erythrocytes, agarose GP-36 (Nakarai Chemicals Ltd., Kyoto, Japan), rabbit anti-human IgG serum (Cappel Laboratories, Cochranville, PA) and sera of guinea pig as complement (Pierce et al., 1971; Gronowicz et al., 1976). The results were expressed as mean numbers of IgG-PFC/culture well (1 × 10⁶ PB-MNC or 5 × 10⁴ B cells) in triplicate cultures.

**Statistical analyses**

Data were analyzed by Student's t-test and linear regression.

**Results**

**Basic studies on the response to SAC by PB-MNC from patients with AITD and normal controls**

The dose response to ISC generation by PB-MNC stimulated with SAC in 4 patients with Graves' disease and 4 normal controls is shown in Fig. 1. As can be seen, the optimal concentration of SAC was 0.01% for both groups. The response to SAC (0.01%) by PB-MNC from 4 patients with AITD was approximately maximal on the 5th day of culture (Fig. 2), and the day of approximately maximal SAC (0.01%) response in 4 normal controls was the same 5th day of culture. Therefore, we studied the ISC response of PB-MNC from patients with AITD and from normal controls at the SAC level of 0.01%, and on the 5th day of culture.

**Fig. 1.** Dose-dependent response to ISC generation by PB-MNC following SAC stimulation in patients with Graves' disease and normal controls. PB-MNC (1 × 10⁶) were cultured in vitro with various concentrations of SAC at 37°C for 5 days. ISC were detected by a reverse PFC assay using protein A-coated sheep erythrocytes. Results indicate the mean ± SD of PFC by PB-MNC in 4 cases in each group.
ISC response by PB-MNC stimulated with SAC in patients with AITD and normal controls

As shown in Fig. 3, ISC response of PB-MNC without stimulation was hardly observed in normal controls and patients with AITD. ISC response of PB-MNC following stimulation with SAC was markedly increased in normal controls. However, this SAC response was significantly observed in 4 cases of AITD.
decreased in patients in the hyperthyroid state of Graves’ disease and with Hashimoto’s thyroiditis. There was little difference in SAC response between in patients in the euthyroid state of Graves’ disease and normal controls. SAC response in the patients in the hyperthyroid state of Graves’ disease was significantly lower than that in the euthyroid state of Graves’ disease.

\[ r = -0.5239 \]
\[ p < 0.05 \]
\[ n = 17 \]

Fig. 4. Correlation between SAC stimulated ISC production from PB-MNC and TRAb in patients with Graves’ disease.

Fig. 5. Effect of IL-6 stimulation on ISC production from circulating B cells in patients with AITD and normal controls. Purified B cells (5 × 10⁴) were cultured in vitro in the presence (●) or absence (○) of IL-6 at 37°C for 5 days. ISC were detected by a reverse PFC assay using protein A-coated sheep erythrocytes. Each point shows the mean PFC/5 × 10⁴ B cells in triplicate cultures from individual persons. The solid horizontal lines and brackets indicate the mean ± SD for each group. Data were analyzed by Student’s t-test.*; p < 0.05; NS, not significant. Although not shown in the figure, the results of statistical analysis for each group related to spontaneous ISC generation are identical with the results of statistical analysis between each group related to IL-6 response shown in this figure.
**Correlation between SAC stimulated ISC response by PB-MNC and other immunological indices in patients with AITD**

The ISC response by PB-MNC following SAC stimulation exhibited a reciprocal relationship to TRAb activity in patients with Graves' disease (Fig. 4), but there were no significant relationships between SAC response and other immunological indices such as the titer of MCHA or TGHA in patients with AITD.

**Spontaneous ISC response and the effect of IL-6 stimulation on ISC response from B cells in patients with AITD and normal controls**

In order to further assess the immunological functions of AITD, we used purified B cells. As shown in Fig. 5, some ISC response without SAC stimulation was observed in patients with AITD. The spontaneous ISC response was further enhanced by IL-6 in B cells from patients in the hyperthyroid state of Graves' disease and with Hashimoto's thyroiditis, although the scatter varied widely between cases. The spontaneous ISC response was not enhanced in B cells from patients in the euthyroid state of Graves' disease. No significant ISC response was observed in normal controls even in the presence of IL-6. No significant relationships were observed between these IL-6 responses and other immunological indices in patients with AITD.

**Discussion**

Hyperreactivity of B cells which produce large amounts of Ig and various kinds of thyroid-specific autoantibodies is one of the most remarkable features of patients with AITD. To date, several investigators have attempted to elucidate the mechanism of autoantibody production by B cells in patients with AITD utilizing the reverse hemolytic plaque assay (Noma et al., 1982; Iwatani et al., 1987). In the present study, we analyzed the immunological functions of circulating B cells in patients with AITD by stimulation with SAC or with IL-6 using the reverse hemolytic plaque assay in vitro.

We demonstrated that the response to ISC by PB-MNC following stimulation with SAC was significantly decreased in patients in the hyperthyroid state of Graves' disease and with Hashimoto's thyroiditis as compared with that of normal controls. Previous investigations of the mitogenic action of SAC on human B cells showed that the response is independent of T cells and that the stimulatory component is protein A on the bacterial wall. The B cells induced by SAC are considered to be resting B cells (Romagnani et al., 1978; Falkoff et al., 1982). Thus, it can be considered that the decreased response of B cells to SAC indicates a decrease in the number of resting B cells and an increase in the number of activated B cells. We should consider the participation of T cells in the response by PB-MNC to ISC following stimulation with SAC, because the differentiation of B cells into Ig-secreting cells in response to SAC was found to require T cells or T cell-derived differentiation factors (Falkoff et al., 1982). Nevertheless, in our results, decreased SAC response was considered to be mainly due to the reduction in the number of resting B cells, because it is reported that the major defect in T cell function is a defect in the suppressor T cell in AITD (Volpé, 1985), and this leads to concern that suppressor T cell defect induces excessive Ig-producing cell generation by permitting the intensification of helper T cell function, so that one may expect stronger response to SAC than that of normal controls which is contrary to our results showing that decreased ISC response was obtained in response to SAC in patients with AITD. Therefore, our findings suggest
that in organ-specific autoimmune diseases such as AITD, some of the circulating B cells are nonspecifically activated.

These results were similar to the case of SLE, one of the systemic autoimmune diseases, about which Tanaka et al. reported that circulating B cells from SLE patients markedly proliferated and produced Igs without any stimulation. However, following stimulation with SAC, B cell response in SLE patients was not enhanced, but was rather decreased, and they suggested that SLE B cells are previously activated and can spontaneously proliferate and differentiate into Ig-secreting cells in an in vitro culture (Tanaka et al., 1988).

We also found that the response to ISC by PB-MNC following stimulation with SAC exhibited a reciprocal relationship to TRAb in patients with Graves' disease. This finding may mean that the higher the TRAb activity is the lower the SAC response would be, namely B cells are activated in the immunologically active state in patients with Graves' disease.

We showed that purified B cells produce ISC spontaneously in patients in the hyperthyroid state of Graves' disease and with Hashimoto's thyroiditis. This spontaneous ISC response was further enhanced by stimulation with IL-6. Since IL-6 has been shown to act in the late stages of B cell differentiation leading to the Ig-secreting cells (Hirano et al., 1986; Hirano et al., 1987), these results suggest that some of the circulating B cells are previously activated and proliferated in AITD.

There have been several experimental reports which suggest an effect of increased amounts of thyroid hormones in the immune response (Alquist, 1976; Volpé, 1985). Furthermore, Volpé et al. suggested that hyperthyroidism does indeed have an adverse effect on the number of generalized suppressor T cells, and presumably their function which ultimately returns to the normal level after the hyperthyroidism itself has been controlled, whether or not an organ-specific defect persists (Volpé et al., 1986). However, we could demonstrate the spontaneous activation of circulating B cells not only in patients with Graves' disease but also in patients with Hashimoto's thyroiditis, and these results could not be attributed to excessive thyroid hormone which may induce non-specific B cell activation by disturbing the generalized suppressor T cell function.

Although we did not prepare B cells directly from the thyroid gland itself which is known to be the major site of immunological reactions in AITD, there have been several reports suggesting that the circulating B cells may provide a useful vehicle by which sequential changes occurring at the major sites of autoantibody production, such as the thyroid gland itself, may be examined (Iwatani et al., 1987; Weetman et al., 1985). Therefore, our results may reflect the immunological abnormality of B cells in the thyroid gland itself.

References


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