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Suppression of anti-erythrocyte autoantibody-producing B cells by a physiological IgG-anti-F(ab′)$_2$ antibody and escape from suppression by tumour transformation; a model relevant for the pathogenesis of autoimmune haemolytic anaemia

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SUMMARY

We showed previously that broadly reactive IgG anti-immunoglobulin autoantibodies produced by rats during the immune response suppress the B cell response. We report here on the effect of a similar human antibody on self-reactive human B cells. IgG anti-F(ab′)$_2$ was added to cultures of anti-erythrocyte autoantibody-producing B cells derived from healthy donors. A dose-dependent suppression of the antibody response was obtained (maximum at 1·3 ng IgG/10^6 cells). This effect was competitively inhibited by F(ab′)$_2\gamma$. Autoimmune haemolytic anaemia can be caused by chronic monoclonal B cell proliferation. To reproduce this condition *in vitro* we immortalized B cells with Epstein–Barr virus (EBV) and raised a B cell population with anti-erythrocyte autoantibody activity. These cells were electrically fused with CB-F7 tumour cells and an IgG1 cold-reactive anti-erythrocyte autoantibody-producing B cell line was established. Surprisingly, the tumour cells were not suppressed by IgG anti-F(ab′)$_2$. It is known that anti-immunoglobulins selectively suppress antigen-receptor (AgR)-occupied B cells by a Fcγ-receptor (FcγR)-mediated mechanism. To occupy their AgR, we preincubated the tumour cells with anti-AgR antibody. In spite of this, their susceptibility to suppression was not restored. As shown by rabbit IgG-sensitized ox erythrocyte (EA)-rosetting, this refractoriness was not due to a loss of FcγR. Our experiments delineate a mechanism of peripheral B cell suppression to autoantigens, and show a way of escape from control relevant for the pathogenesis of autoimmune haemolytic anaemia.

Keywords B cells autoimmunity immunoregulation autoantibody immunological tolerance

INTRODUCTION

An enormous antibody diversity is generated within the B cell compartment during its development [1,2]. Randomly generated self-reactive clones are excluded either by functional inactivation (anergy) or by physical elimination (deletion). Whether one or the other mechanism is involved depends on the manner of antigen presentation and possibly on other factors [3–7]. Tolerance induction generally requires a high affinity B cell receptor and a certain antigen concentration [5]. B cells with low affinity [6] or whose membrane immunoglobulins (mIg) are only partially occupied by autoantigens due to low antigen concentrations [4,8–11] escape central tolerance induction and become part of the peripheral B cell repertoire. This process generates one of several autoreactive B cell populations found in healthy individuals.

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Recently, it has been shown that even B cells rendered tolerant in central lymphatic organs can be reactivated in the periphery under certain conditions [12]. This is a second source of autoreactive B cells.

Tolerance induction requires contact between autoantigen and B cells. Many autoantigens are not expressed in central lymphoid organs and thus cannot induce self-tolerance [8,13]. Such autoantigens are 'seen' by B cells for the first time in the periphery. This is a third family of autoreactive B cells.

During the generation of diversity by hypermutation in peripheral lymphoid organs, populations of new B cell specificities including autoreactive lymphocytes arise [14]. Moreover, during this process the affinity of low affinity self-reactive B cells increases [15]. An entire 'wave' of high affinity autoimmune B cells results. This is a fourth population of peripheral self-reactive B cells.

Because of escape from central tolerance induction, or because of newly generated autoreactive B cells, the immune

system must have 'invented' peripheral mechanisms of tolerance induction [4,11,16].

We showed previously that during the immune response to alloantigens, rats develop a suppressive IgG anti-immunoglobulin autoantibody which regulates the B cell response [17,18]. Similar antibodies have been described in man [19–21]. Our studies focused on a human IgG antibody which recognizes a conserved domain of the $F(ab')_2\gamma$ region, therefore termed IgG-anti-$(Fab')_2$. The current experiments addressed the question, whether this physiological antibody regulates peripheral autoantibody-producing B cells of healthy donors.

MATERIALS AND METHODS

Cell cultures

Lymphocytes were collected from a healthy volunteer by lymphocytophoresis in a blood cell separator (Fenwal CS-3000) and stored in liquid nitrogen until use. Concomitantly, erythrocytes were collected and frozen. These 'resting lymphocytes' were activated in culture by adding either supernatants of antigen-activated lymphocytes or interleukins.

Stimulation with supernatant. The same volunteer was vaccinated intramuscularly with 75 U of tetanus toxoid (TT) and the anti-TT antibody titre was measured daily. On day 10 peripheral lymphocytes were collected again by cytopheresis and stored in liquid nitrogen. Antigen-activated lymphocytes were cultured (2×10^6/ml per well) and lymphokine-rich supernatants collected (=TT−SN). Various dilutions of TT−SN were added to resting lymphocytes in cultures.

Stimulation with interleukins. A combination of recombinant human interleukins was used for activation of resting lymphocytes (20 U/ml IL-1, 50 U/ml IL-2) (DRG Instruments, Marburg, Germany). Various amounts of IgG anti-$F(ab')_2$ antibody were added to the cultures together with activators. B cell antibody production was determined after 3 days. Each culture was performed on three different days.

Competitive inhibition experiments were carried out with increasing amounts of $F(ab')_2\gamma$ fragments added to activated cells before the IgG anti-$F(ab')_2$ antibody (2 ng/10^6 cells). Control cultures consisted of $F(ab')_2\gamma$ and activator only. In order to occupy the B cells' antigen receptor, 10^6 lymphocytes were incubated for 30 min at 22°C in serum-free medium with 37 μg of goat $F(ab')_2$ anti-human $F(ab')_2$ (Jackson Immunoresearch Lab., West Grove, PA). This antibody reacts with both $F(ab')_2\mu$ and $F(ab')_2\gamma$, and binds to Epstein–Barr virus (EBV)-infected and tumour-transformed B cells as shown by FACS analyses.

Cell cultures were carried out in HB104 serum-free medium (Laboserv Diagnostica, Giessen, Germany).

Detection of antibody production in cell cultures

Plaque-forming cells assay (PFC). Anti-erythrocyte autoantibody production of single B cells was measured in a PFC. Lymphocytes (4×10^4) suspended in 20 μl medium were incubated at 22°C with 6 μl autologous erythrocytes and 25 μl rabbit or human complement. To remove heterophilic antibodies the complement was extensively absorbed with erythrocytes. The number of PFC per 10^6 cultured cells was determined (mean ±s.e.m.). Positive controls consisted of stimulated lymphocytes (100% antibody response) and negative controls of unstimulated lymphocytes (0% response).

ELISA-spot assay (ELISPOT). Total IgM or IgG antibody production of single B cells was examined in the ELISPOT assay. Culture plates were coated with 5 μg/well of goat $F(ab')_2$ anti-human IgM, or with 7·5 μg/well of goat $F(ab')_2$ anti-human IgG (Jackson Immunoresearch). Unspecific binding sites were blocked with PBS + 1% gelatine. Test lymphocytes suspended in serum-free medium were incubated in precoated culture plates for 4 h at 37°C, and thereafter removed. Alkaline phosphatase-conjugated goat anti-human IgG(Fc) or goat anti-human IgM (Medac, Hamburg, Germany) were applied for 1 h at 37°C. Heated low EEO agarose containing 0·4 μg 5-brom-4-chlor-3-indolyl-phosphate and 0·4 ml 2-amino-2-methyl-1-propanol (Sigma Chemical Co., St Louis, MO) was applied to the plates. After 30 min blue spots, indicating single antibody-producing B cells, were counted in a Labovert FS microscope connected to a Microvid computer system (Leitz, Wetzlar, Germany).

IgG anti-$F(ab')_2$ antibody

Detection. Microtitre plates were coated with 1 μg/well of human $F(ab')_2\gamma$ fragments (Jackson Immunoresearch). Remaining active groups were blocked with PBS + 1% gelatine. All test sera were adjusted to a concentration of 0·4 mg IgG/ml and applied to precoated microtitre plates (50 μl/well, triplicates). A reference serum with known IgG anti-$F(ab')_2$ activity was used as positive and PBS as negative control. After incubation, 50 μl of alkaline-conjugated goat anti-human IgG(Fc)(Jackson Immunoresearch) were added. Each step was followed by extensive washing with PBS + 0·05% Tween. Substrate (250 μg *p*-nitrophenyl phosphate disodium/well) (Sigma) was added and the extinction was measured at 405 nm every minute up to 90 min. The test was stopped at an extinction of 0·37 in the positive control.

Separation. The IgG of sera containing anti-$F(ab')_2$ antibodies was separated by two-dimensional high performance liquid chromatography (Mono Q HR, Pharmacia Biosystems, Freiburg, Germany, and Protein G, Selectispher-10, Perstorp Biolytica, Lund, Sweden) [17]. IgG purity was verified by SDS–PAGE. Thereafter, the anti-$F(ab')_2$ antibody was extracted from IgG by affinity chromatography on a $F(ab')_2$-agarose column (Jackson Immunoresearch). The eluate (20 μg/ml) showed an anti-$F(ab')_2$ antibody activity of 0·969. The negative control had an extinction of 0·07.

Transformation and cloning of anti-erythrocyte autoantibody producing B cells of a healthy donor

The B cells of a healthy volunteer were infected with EBV. IgM- and IgG-producing B cells were assessed by immunoglobulin titration of supernatants, ELISPOT, and FACS analysis of cultured cells. Anti-erythrocyte autoantibody production was demonstrated in supernatants by the indirect Coombs test and by PFC formation with autologous erythrocytes. After 169 days B cell hybridomas were produced by electrofusion [22] with CB-F7 heteromyeloma. A stable B cell line was established which produced a monoclonal IgG1 cold-reactive anti-erythrocyte autoantibody.

FACS analysis

One million B cells were incubated in serum-free medium with 37 μg goat $F(ab')_2$ anti-human $F(ab')_2$-FITC (Jackson Immunoresearch), washed extensively and analysed in a FACScan

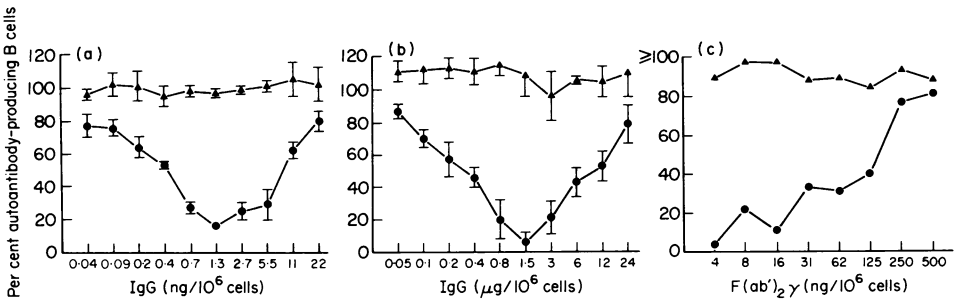

Fig. 1. Suppression of anti-erythrocyte autoantibody-producing B cells with IgG anti-F(ab')$_2$ antibody and abrogation of suppression with F(ab')$_2\gamma$ fragments. Various amounts (abscissa) of (a) affinity-purified IgG anti-F(ab')$_2$ (●) or (b) serum IgG with anti-F(ab')$_2$ activity were added to stimulated lymphocytes. IgG without anti-F(ab')$_2$ (▲) activity served as control. (c) Increasing amounts of F(ab')$_2\gamma$ fragments (abscissa) were added before IgG anti-F(ab')$_2$ antibody to stimulated cells. Controls consisted of cultures with F(ab')$_2$ only. ▲, F(ab')$_2\gamma$; ●, anti-F(ab')$_2$ + F(ab')$_2\gamma$.

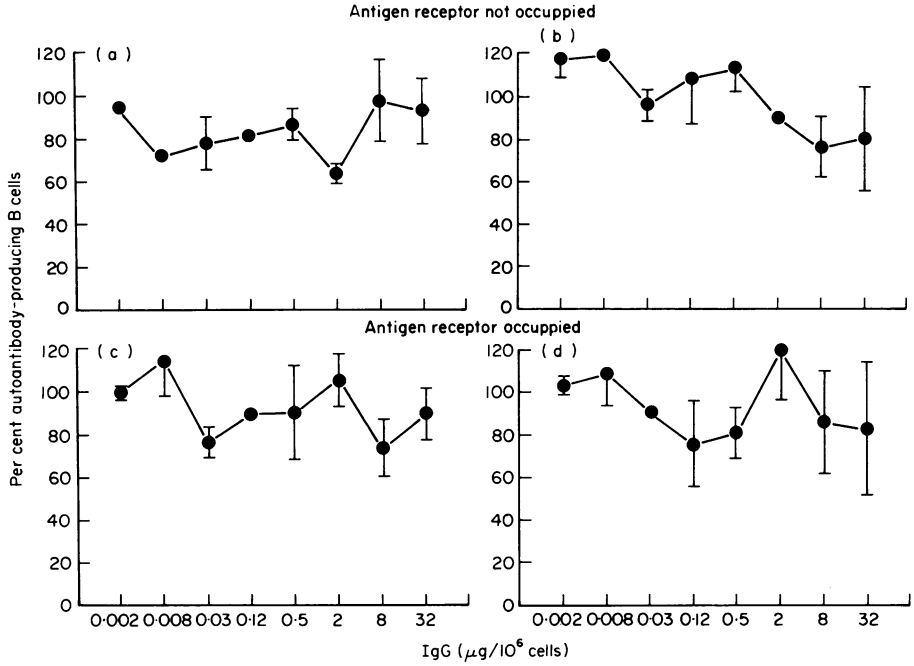

Fig. 2. Effect of IgG anti-F(ab')$_2$ antibody on Epstein–Barr virus (EBV) cells and tumour cells. (a) EBV-transformed B cells or (b) tumour B cells were incubated with various amounts of IgG anti-F(ab')$_2$ antibody (abscissa) and the number of anti-erythrocyte autoantibody-producing cells was determined. Positive controls (100% antibody response) were cultures without IgG anti-F(ab')$_2$. (c) EBV cells or (d) tumour cells were preincubated with goat F(ab')$_2$ anti-human-F(ab')$_2$ antibody (antigen receptor occupancy) and cultured with various amounts of IgG anti-F(ab')$_2$. Positive controls consisted of antigen receptor-occupied cells.

flow cytometer (Becton Dickinson, Sunnyvale, CA). Dead cells were excluded by staining with ethidium bromide.

Rosette formation with IgG antibody-coated ox erythrocytes

Fcγ receptors (FcγR) on B cells were detected by rosette formation with rabbit IgG-sensitized ox erythrocytes (EA) as described elsewhere [23]. Briefly, ox erythrocytes were incubated at 37°C for 1 h with increasing amounts of rabbit IgG anti-ox erythrocyte antibody (Nordic, Tilburg, The Netherlands), washed, and adjusted to 2×10^8/ml in gelatin veronal buffer supplemented with calcium and magnesium. One million test lymphocytes suspended in 20 μl culture medium supplemented with 10% fetal calf serum (FCS) were mixed with 100 μl ox EA, and centrifuged. The rosette pellet was resuspended by gentle shaking, fixed with 3% glutaraldehyde, stained with 0·75% trypan blue and inspected under sealed coverslips. The percentage of rosette forming lymphocytes (%RFC) was determined.

RESULTS

Peripheral B cells of healthy persons can be driven to anti-erythrocyte autoantibody production by physiological activators

The stimulation of cells derived from three healthy donors with interleukins resulted in a vigorous anti-erythrocyte autoantibody response of 9452 ± 192 PFC/10^6 cells (unstimulated cells $= 987 \pm 229$ PFC/10^6 cells).

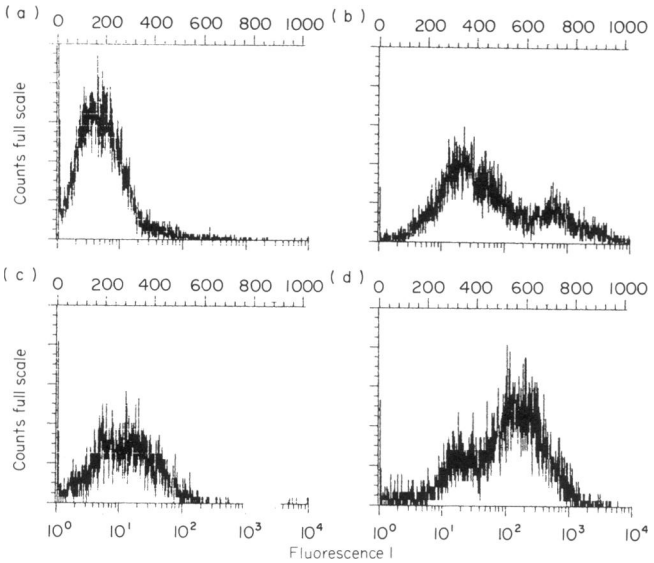

Fig. 3. Binding of goat $F(ab')_2$ anti-human-$F(ab')_2$ antibody to Epstein–Barr virus (EBV) cells and tumour cells. EBV cells or tumour B cells were incubated with FITC-labelled goat $F(ab')_2$ anti-human-$F(ab')_2$. The FACS histogram shows the log of green fluorescence (abscissa) *versus* cell number (ordinate) of (a) unlabelled and (b) labelled EBV cells, and (c) unlabelled and (d) labelled tumour cells.

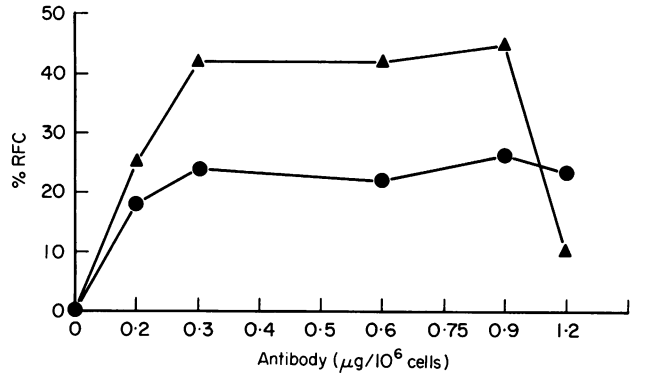

Fig. 4. EA rosette formation of tumour B cells. Tumour B cells and normal peripheral lymphocytes (control) were rosetted with ox erythrocytes coated with increasing amounts (abscissa) of rabbit IgG anti-ox erythrocyte antibody (EA). Some agglutination of erythrocytes was noted at 1·2 μg antibody/10^6 cells. The percentage of rosetting lymphocytes (% RFC) is shown on the ordinate. ▲, Tumour cells; ●, normal lymphocytes.

Since lymphokines are produced during the course of every physiological immune response, we expected that supernatants of antigen-activated lymphocytes would also activate autoantibody-producing cells. One of the test persons was immunized with TT. Ten days later, at the time of maximum anti-tetanus antibody production, lymphocytes were collected by cytopheresis, put in culture without any activator, and lymphokine-rich supernatants (TT−SN) were collected. The stimulating capacity of these supernatants on resting lymphocytes derived from the same donor was tested. Similar to interleukins, TT−SN induced anti-erythrocyte autoantibody production up to 9200 PFC/10^6 cells.

To better appreciate the *in vivo* relevance of our test system, rabbit complement was replaced in the plaque-forming cell assay with autologous human complement. A similar degree of erythrocyte lysis was achieved with heterologous or autologous complement.

Autoantibody-producing B cells are suppressed by a physiological IgG anti-$F(ab')_2$ antibody

Based on our experience in the rat system [17,18], we analysed in an autologous human test system whether a physiological IgG anti-$F(ab')_2$ antibody has a regulatory effect on self-reactive B cells.

The IgG fraction was separated from serum of a healthy donor. In a second step, anti-$F(ab')_2$ antibody was extracted by affinity chromatography. Various amounts of affinity-purified antibody (Fig. 1a) were added to lymphocytes concomitantly stimulated with TT−SN. The lymphocytes were derived from the IgG donor as well as from two other donors. With increasing amounts of anti-$F(ab')_2$ antibody the anti-erythrocyte autoantibody production of B cells gradually decreased. Maximum suppression was obtained at 1·3 ng/10^6 cells. Interestingly, further increase of the antibody concentration caused the suppressive effect to disappear. The negative control (IgG without anti-Fab'_2) had no effect. *In vivo* the anti-$F(ab')_2$ antibody is part of the serum IgG fraction. To determine whether in this 'natural' form the anti-$F(ab')_2$ is active, the serum IgG fraction containing the anti-$F(ab')_2$ antibody was tested. As shown in Fig. 1b, the antibody maintained its suppressive activity, although the optimal dose was far higher than that of the affinity-purified fraction (1·5 μg/10^6 cells).

The suppressive activity of anti-$F(ab')_2$ antibody is competitively inhibited by $F(ab')_2\gamma$

To test whether the suppressive activity of IgG anti-$F(ab')_2$ can be competitively inhibited, increasing amounts of $F(ab')_2\gamma$ fragments were added to the cultures. A dose-dependent inhibition was noted (Fig. 1c).

Susceptibility to suppression is lost by tumour transformation of autoreactive B cells

Although it is known that EBV can induce autoimmune haemolytic anaemia, the exact mechanism leading to the disease remains obscure [24]. Peripheral B cells of the previously tested volunteer were EBV-infected. A polyclonal IgG- and IgM-producing B cell population was obtained. Anti-erythrocyte autoantibody production of EBV cells was detected in the PFC assay. To our surprise, these autoantibody-producing B cells were not suppressed by anti-$F(ab')_2$ antibody (Fig. 2a). We also measured their total IgM and IgG production. No evidence for suppression was found (data not shown).

Our previous studies demonstrated that anti-immunoglobulins selectively suppress antigen receptor (AgR)-occupied B cells [18]. Conceivably, cells kept in culture for a longer time lose their receptor-bound autoantigen. It is generally accepted that anti-immunoglobulin antibodies directed against B cell AgR are able to 'replace' the antigen [25]. To exclude the possibility that the absence of suppression was caused by lacking AgR occupancy, the EBV cells were incubated before culture with goat $F(ab')_2$-anti-human-$F(ab')_2$. This antibody was shown by FACS analysis to bind to the cells (Fig. 3a,b). However, the AgR-occupied B cells did not regain their susceptibility to suppression (Fig. 2c).

Subsequently, IgG-producing EBV cells were electrically fused with CB-F7 tumour cells and cloned. A monoclonal cell line which produced a cold-reactive IgG1 anti-erythrocyte autoantibody was obtained. Whereas it is not certain whether EBV-induced B cell proliferation is responsible for autoantibody production in acute anaemia, it is known that tumour transformation of an autoimmune B cell clone may cause haemolytic anaemia [26]. Therefore, it was of interest whether the autoreactive tumour cells could be regulated by IgG anti-$F(ab')_2$ antibody. The cells were not suppressible (Fig. 2b). Even after AgR occupancy with antibody (Fig. 3c,d) the tumour cells remained refractory to suppression (Fig. 2d). The same results were obtained by measuring the total antibody production by ELISPOT assay (data not shown).

The binding of anti-immunoglobulin antibody (Fig. 3) demonstrates the presence of immunoglobulins on the membrane of tumour cells, whereas EA rosette formation demonstrates the presence of FcγR (Fig. 4).

DISCUSSION

Roosnek & Lanzavecchia showed that anti-immunoglobulin-producing B cells capture TT- anti-TT immune complexes and present them to specific T cells [27]. The T cells release lymphokines which trigger anti-immunoglobulin-producing B cells. This explains earlier findings that anti-immunoglobulin-producing B cells are activated during the immune response both to TT and to other antigens [28,29], leading to an increased anti-immunoglobulin serum titre.

Our data show that during the immune response to TT mediators are generated which are able to stimulate anti-erythrocyte autoantibody-producing B cells. Therefore, the immune system must have developed contraregulatory mechanisms that prevent autoaggression.

Since B cells bear immunoglobulins on their membrane, anti-immunoglobulin antibodies may bind to them and regulate their response. Many previous experiments showed that heterologous anti-immunoglobulin antibodies suppress the B cell response [30]. Furthermore, it has been shown that the immune system of man and animals produces anti-immunoglobulin autoantibodies [20,21,31–33]. In human sera, some of these antibodies were termed pepsin or papain agglutinators, according to their specificity for $F(ab')_2$ or Fab fragments [20,21]. We hypothesized that IgG anti-$F(ab')_2$ produced during the anti-tetanus immune response as well as during responses to other antigens prevents the activation of autoreactive B cells. The present findings show that this antibody is fully capable of suppressing anti-erythrocyte autoantibody-producing B cells. Importantly, the suppression was perfectly reproducible with the whole IgG fraction containing the regulatory antibody, indicating that the antibody is active in its natural environment. There are several possible explanations for the fact that the antibody is not blocked by free IgG in the serum: (i) the antibody has a low affinity for IgG. This is supported by the high $F(ab')_2\gamma$ concentration required for competitive inhibition. Because the low-affinity antibody is blocked only partially by serum IgG, unbound antibody is still available for suppression; (ii) the antibody may have a stronger affinity for mIg than for serum immunoglobulins; (iii) suppression may be mediated by immune complexes formed between anti-$F(ab')_2$ autoantibody and autologous IgG. In this case, unbound antibody is not required for B cell inactivation.

In addition to other described control mechanisms [7,11,16] our data indicate a new way of suppressing self-reactive peripheral B cells. Accordingly, a certain basic IgG anti-$F(ab')_2$ antibody titre found in all individuals provides an efficient means for continuous suppression of self-reactive B cells. Increased anti-$F(ab')_2$ titres prevent the activation of autoreactive B cells by stimulators produced during the immune response to foreign antigens.

It is interesting to note that the human antibody described here presents a similar dose response curve as the previously described rat antibody: above a certain concentration it loses its suppressive effect [17]. The mechanisms leading to this type of response were commented on extensively elsewhere [18].

Although much has been learned about tolerance induction from experiments in transgenic mice, the mechanism of escape from tolerance leading to autoimmune diseases remains obscure. The results reported here point out two ways in which self-reactive B cells might escape from suppression: either by a decrease of regulatory antibody titre beneath a critical level, or by a loss of B cell susceptibility to suppression. Silvestris *et al.* [34] showed that patients with severe systemic lupus erythematosus (SLE) have high levels of anti-DNA and low levels of anti-$F(ab')_2$ antibodies, while patients with quiescent SLE present low levels of anti-DNA and high levels of anti-$F(ab')_2$. In the light of our data, these findings suggest that a decrease of suppressive anti-$F(ab')_2$ antibodies weakens the control over autoreactive B cells, leading to an exacerbation of autoimmunity. In the current series of experiments we analysed the susceptibility of autoimmune B cells to suppression following tumour transformation. By transforming an anti-erythrocyte autoantibody-producing B cell into a tumour cell, we reproduced under laboratory conditions the process which leads to haemolytic anaemia in patients with chronic monoclonal lymphoproliferation [26]. Our data demonstrate that if an autoreactive B cell is transformed into a tumour cell, it escapes from anti-$F(ab')_2$-induced suppression.

We showed previously that suppression by anti-immunoglobulins requires the presence of mIg and FcγR on the B cell membrane [18]. In the current experiments, both immunoglobulins and FcγR were present on the tumour-transformed cells. Thus, the tumor cells' refractoriness to suppression could not have been caused by a loss of their receptors.

The data presented in this study describe a control mechanism of peripheral autoantibody-producing B cells, and circumvention of control by tumour transformation. These findings contribute to a better understanding of the pathogenesis of autoimmune haemolytic anaemia.

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