EFFECTS OF ADMINISTRATION OF CYCLOPHOSPHAMIDE AND ANTI-THYMOCYTE-GLOBULIN ON SERUM LEVELS OF COMPLEMENTS C1q AND C3 IN MICE

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Abstract: Serum levels of the initiating complement component (C1q) in the classical pathway and those of an essential triggering component (C3) in the altemative pathway were quantified immunochemically in parallel with those of the immunonoglobulin G (IgG) in BALB/c mice, each of which was given a single dose of cyclophosphamide (CY) and/or rabbit anti-mouse-thymocyte-globulin (anti-TG) intraperitoneally. In the CY-treated mice, both C1q and IgG levels were significantly suppressed from 2 through 14 days after the treatment; such suppression was more conspicuous in male than in female mice. In contrast, C3 levels were markedly increased in these mice of both sexes from 2 through 7 days after the same treatment. In anti-TG-treated mice of both sexes, only C1q levels were significastly suppressed from 2 through 10 days after the treatment; however, neither their C3 nor IgG levels were much influenced. Both T and B lymphocytes in peripheral blood of these mice were significantly diminished: CY affected more severely B cells; in contrast, anti-TG had a more severe influence on T cells. These results suggest that there may be some metabolic and/or developmental interrelationships among serum C1q levels, functions of immune lymphocytes, and their producing immunoglobulins and/or lymphokines.

Index Terms

IgG and IgM, cyclophosphamide, anti-mouse-thymocyte-globulin, C1q receptor EDTA-blood

INTRODUCTION

The complement system consists of a set of some 20 distinct proteins, forming a complex biological system which fulfils various functions. This system not only plays important roles in host defence mechanisms but also is implicated in disease processes involving cyotoxic and immune complex-mediated hypersensitivites. For modulating these diverse biological activities, the system must be activated in the classical[1] or in the alternative pathway[2]. C1q is the triggeming component of the classical pathway, and it is a subcomponent of the first component (C1); C1 is composed of three different subcomponents, namely C1q, C1r and C1s. This subcomponent can interact not only with some classes of immunoglobulin(IgG and IgM)in the form of antigen-antibody complexes but also with non-antibody-substances, e. g. polyanions, polycations, etc. C3 is the third component of complement actually reacts fourth, and plays the

central role of the classical pathway. In addition, the C3 participates in the alternative pathway as an essential triggering component together with factots B and D. C3 interacts mainly with non-antibody-substances such as yeasts or bacterial lipopolysaccharide.

Serum C1q is mainly produced in columnar cells of the small intestine[3] and/or in the monocyte-macrophage lineage[4], and its levels have been reported to correlate with serum IgG levels in patients with a variety of immunoglobulin anomalies[5),6)], as well as in normal mice among vamious strains[7]. Several reports have shown that various types of cells possess specific receptors for C1q (C1qR)[8),9)], and have suggested that this subcomponent plays a potential role in the regulation of B cell functions. In fact, recent *in vitro* studies have shown that C1q down-regulates the secretion of IL-1-like activity by B lymphoblastoid cell lines[10] and induces their anti-proliferative response[11] probably through the interaction of the collagenous tail[12] of C1q and C1qR. It has been also shown that C1q significantly augments immunoglobuin production by human B lymphocytes in the presence of the T lymphocytes, perphaps through C1qR[13] and in the absence of T cells most likely at the level of glycolation or secretion of immunoglobulin molecules[14]. C3 is synthesized by liver parenchymal cells[3)15)], and by the monocyte-macrophage lineage[8)16)17)]. In the early 1970s a regulatory link between this C3 and the production of antibody was also postulated[18)-20)]. Other complement components have been shown to have immunoregulatory functions, including C3a, which suppresses, and C5a and its fragment, which enhances *in vitro* antibody formation[21]. In addition, C5a has been shows to stimulate IL-1 production by monocytes[22]. Thus immunoregulatory links of C1q, C3 and its fragment, and of C5a have been gradually well analyzed *in vitro*. However, *in vivo* studies on immunoregulatory mechanisms by complement components are still largely limited.

In this study, we have, therefore, artificially regulated B and T cell functions in mice by *in vivo* administration of CY and/or anti-TG, and measured immunochemically their serum levels of C1q and C3, which should reflect the sum of their biosynthesis and catabolism, in parallel with those of the serum IgG. It is reported that CY suppresses both T and B cell populations, but that anti TG suppresses only the T cell lineages. In this manuscript, we document our findings that both the reagents suppressed C1q levels and that C3 levels fluctuated only in CY -treated mice.

MATERIALS AND METHODS

Animals and their treatment with CY or anti-TG

BALB/c mice of both sexes (from 2 to 6 months old, 20-25 g body weight, total 212 mice) were used. The mice, originally a gift from the Center for Laboratory Animals in the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan), have been maintained by sister-brother mating in our laboratory. Each of these mice was given a single intraperitoneal dose of CY (20 mg/ml in sterile water in doses of 300 μg/ml or 400 μg/ml body weight ; Sigma Chemical Co St. Louis, MO, USA) or a sterile rabbit anti-TG (0.2 ml) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as indicated in the instruction manual for this antiserum by the company. The *in vivo* potency of this anti-TG in abrogating T cell functions was established by the supplier in prolonging the survival of allograft skin. Each of the control mice was given a single intraperitoneal dose of 0.2 ml sterile saline or rabbit IgG (10 mg/ml in saline ; Sigma

Chemical Co.). Several mice of each sex were sacrificed and their blood was obtained by cardiac puncture after anesthesia at 2,7 and 14 days after the adminstration of CY or saline, and at 1,3,5,7 and 10 days after the treatment with anti-TG or rabbit IgG. A part of the blood of each mouse was collected in 0.4 ml of isotonic 0.1 M EDTA (pH 7.4) (EDTA-blood), and exact blood volume collected was calculated by measuring the weight. The remaining blood without adding EDTA was allowed to clot at 37℃ for 60 min and then left at room temperature for 60 min. Serum was separated at 4℃ by centrifugation at 2000 g for 15 min.

Immunological quantitation of C1q, C3 and IgG

This was carried out using a modification of the single radial immunodiffusion described by Mancini *et al*[23]. Rabbit monospecific antiserum to mouse C1q was prepared according to the method previously described[24]. Goat antisera to mouse C3 and rabbit antisera to mouse IgG were purchased from CAPPEL Organon Teknika Corp. (West Chester, PA, USA) and from Miles Laboratories, Inc. (Elkhard, IN, USA) respectively. These antisera were used after appropriate dilutions.

Measurement of blood leukocyte, T and B lymphocytes

Total counts of circulating leukocytes were made in a haemocytometer chamber by using the EDTA-blood obtained as described above after mixing it 1 : 9 with a Turk solution. The lymphocyte rich fraction was obtained by overlaying each EDTA-blood onto a lymphohyte separation solution (M-SMF with the specific gravity 1.090 ; Japan Immunoresearch Laboratories Co. Ltd., Takasaki, Japan), and after removal of erythrocytes by centrifugation at 1200 g for 20 min.

T lymphocytes in the lymphocyte rich fraction were measured by essentially the same Trypan blue dye-exclusion cytotoxic testing as described by Raff and Owen[25], but scaled down by using poly-L-lysine coated Terasaki tissue culture plates (Becton Dickinson and Company, Lincoln Park, NJ, USA). The antiserum reagent used was an AKR mouse monoclonal antibody to CBA Thy-1.2 thymocytes (Thy-1.2 F7D5 monoclonal IgG cytotoxic antibody ; SeroTec, Kidlington, Oxford, England) after heat inactivation at 56℃ for 30 min. Fresh guinea pig serum, absorbed with BALB/C mouse erythrocytes, was used as a source of complement. T lymphocyte counts were expressed as percentage of dead cells stained with Trypan blue to the sum of dead and live lymphocyte populations. For B lymphocyte counts, cellsin the lymphocytes rich fraction were washed 3 times with cold RPMI 1640 (Flow Laboratories, North Ryde, N. S. W., Australia) and incubated in the dark for 30 min at 4℃ with a 1 : 20 dilution of the fluorescein isothiocyanatelabelled $F(ab')_2$ fragment of goat anti-mouse IgM (CAPPEL Organon Teknika Corp.). The cells were washed 3 times with cold phosphate-buffered saline, fluorescent B lymphocytes were counted under a fluorescencè microscope. Percentages of B lymphocytes to cells in the lymphocyte rich fraction were also calculated.

Statintical analysis of data

Experimentally measured values in each group were expressed as the mean±one standard deviation (S. D.). The statistical significance of the difference of the means of C1q, C3, and IgG

levels in CY-, anti-TG- and rabbit IgG-treated mice from those in saline-treated mice was calculated by Behrez-Fisher z test because of the unequality of each variance. The degree of the significance was tested with a t table and probability levels(P) were calculated. These statistical analysis methods have been described by Snedecor and Cochran[26].

RESULTS

Effect of CY on serum levels of C1q, C3 and IgG, and on blood T and B lymphocyte counts

Total 88 BALB/c mice (46 male and 42 female) were used. Each of these mice was treated with a single intraperitoneal dose of CY (300 μg/g or 400 μg/g body weight) or saline. On 2,7 or 14 days after the treatment, several mice were sacrificed and their serum C1q, C3 and IgG, and peripheral blood leukocytes, T and B lymphocytes were measured.

The mean values and the S. D. of C1q, C3 and IgG levels in each group according to sex and days after treatment were calculated and are presented in Fig. 1. In the CY-treated mice, both C1q and IgG levels were highly suppressed : the mean levels of C1q and of IgG corresponded to 44-68% and to 38-54% of those of their respective saline-treated mice, respectively. Such suppression was more conspicuous in male than in female mice, and also more conspicuous in the mice treated with the higher dose of CY. The suppression was statistically significant in male mice from 2 through 14 days ($p<0.001$), and significant in female only on 14 days ($p<0.01$) after the treatment. In contrast, C3 levels in the CY-treated mice of both sexes were

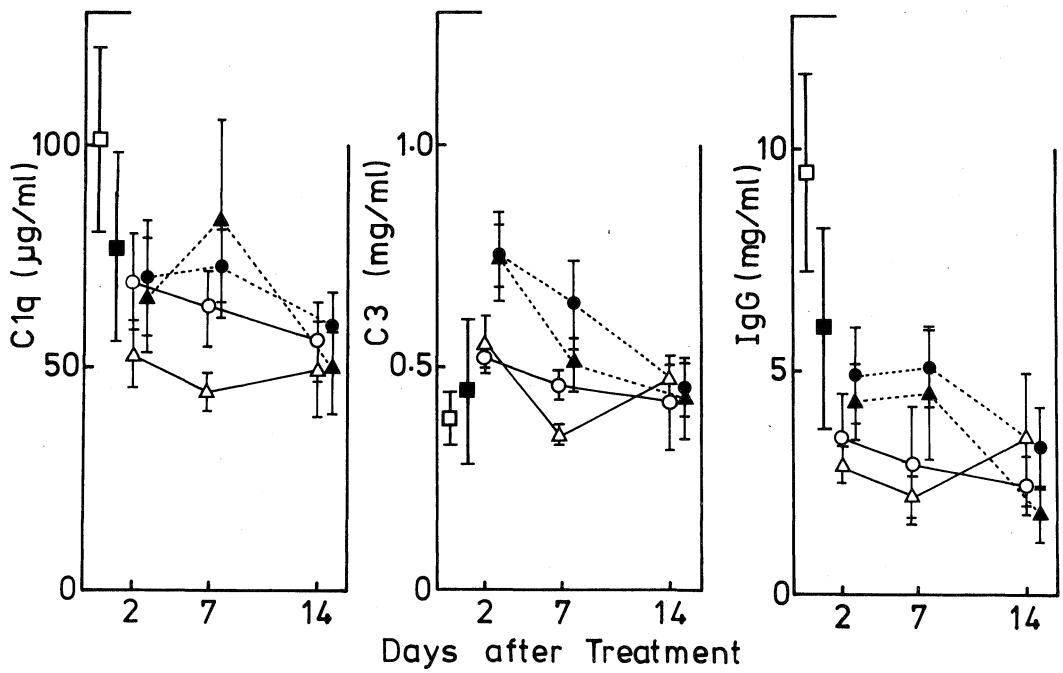

Fig. 1. Serum C1q C3 and IgG levels in BALB/c mice on 2, 7 and 14 days after cyclophosphamide (CY) -treatment. Values are represented as the mean ±1 Standard devdation. □ : saline-treated male controls ; ■ : saline-treated female controls ; ○ : 300 μg/g CY-treated male mice ; ● : 300 μg/g CY-treated female mice ; △ : 400 μg/g CY-treated male mice ; ▲ : 400 μg/g CY-treated female mice.

significantly increased to 142%-169% of the levels of their respective saline-treated control on 2 days ($p<0.01$) and on 7 days ($p<0.05$) after treatment.

The mean values and the S. D. of blood leukocyte counts, T and B lymphocyte counts were calculated and presented in Table 1. In the CY-treated mice in both sexes, total blood leukocyte counts were highly suppressed to 8%-63% of those of their respective saline-treated controls from 2 through 7 days after the treatment. The suppression was more conspicuous in male ($P<0.001$) than in female ($P<0.01$) mice and more conspicuous in the mice with 400 μg/g CY ($P<0.001$) than in those with 300 μg/g CY ($P<0.01$). In the CY-treated mice, percentages of B lymphocytes to cells in the lymphocyte rich fractions were also markedly reduced to 12%-76% of those of respective saline-treated controls through the entire period tested. Such reduction was also more conspiuous in male than in female mice and in the 400 μg/g CY-treated mice than in the 300 μg/g CY-treated mice. Percentage of T lymphocytes slightly increased in the CY-treated mice, but such increase was not statistically significant, and numbers of T cells were actually reduced.

Effect of anti-TG on serum levels of Clq, C3 and IgG, and on blood T and B lymphocyte counts

Total 124 BALB/c mice (63 male and 61 female) were used. Each of these mice was treated with a single intraperitoneal dose of rabbit anti-TG, rabbit IgG or saline. On 1, 3, 5, 7, and 10 days after the treatment, several mice were sacrificed and their serum Clq, C3 and IgG, and

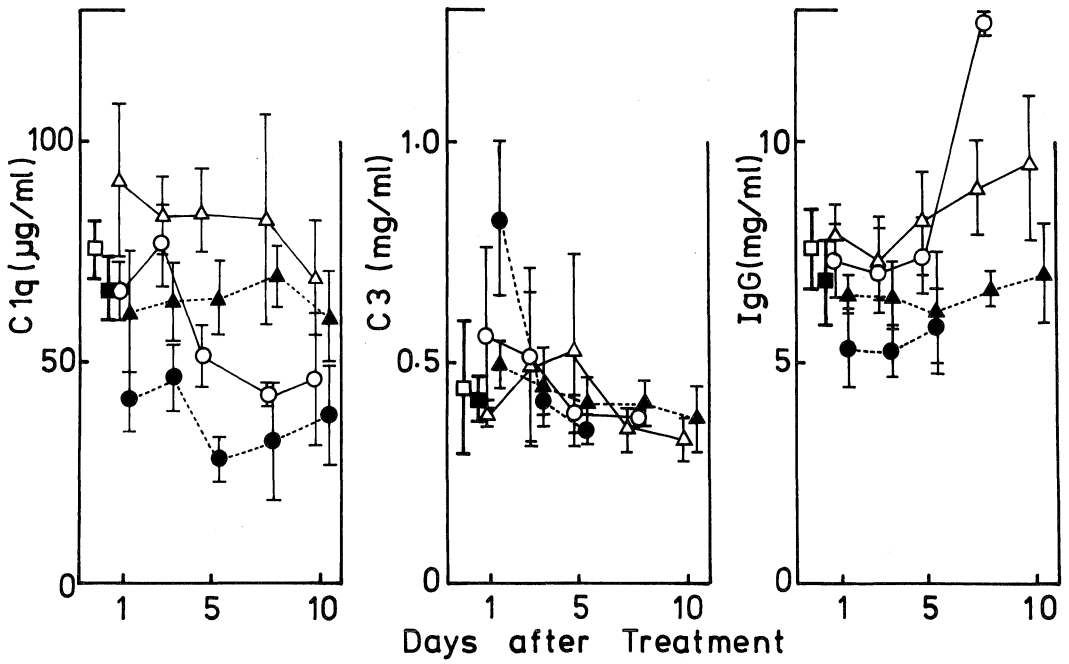

Fig. 2. Serum Clq, C3 and IgG levels in BALB/c mice on 1, 3, 5, 7 and 10 days after anti-thymocyte-globulin (anti-TG)-treatment. Values are represented as the mean ±1 standard deviation. □ : saline-treated male mice ; ■ : saline-treated female mice ; ○ : anti-TG-treated male mice ; ● : anti-TG -treated female mice ; △ : rabbit IgG-treated male mice ; ▲ : rabbit IgG-treated female mice.

peripheral blood leukocytes, T and B lymphocytes were measured. The mean values and the S. D. of C1q, C3 and IgG levels in each group accroding to sex and days after the treatment were calculated and are presented in Fig. 2. In the anti-TG-treated male mice, C1q levels were suppressed to 55-67% of that of the saline-treated male mice ($p<0.001$) from 5 through 10 days after the treatment. In the anti-TG-treated female mice, C1q levels were suppressed to 41-70% of that of the saline-treated female mice on 1 day ($p<0.01$) and on 3 days ($p<0.05$) and 5-10 days ($p<0.001$) after the treatment. In these female mice, IgG levels were also slightly suppressed to 77%-86% of those of the saline-treated female mice. C3 levels of the anti -TG-treated female mice and IgG levels of the anti-TG-treated male mice highly increased on 1 day and on 7 day after treatment respectively. In the rabbit-IgG-treated mice, no significant deviatitons were observed in either C1q, C3 or in IgG levels from those of their respective saline -treated controls.

The mean values and the S. D. of blood leukocyte counts, T and B lymphocyte counts were calculated and presented in Table 2. In the anti-TG-treated mice of both sexes, total leukocyte counts were highly suppressed to 8%-73% of those of their respective saline-treated mice from 1 through 7 days after the treatment. The suppression was highly significant on 1-3 days ($p<0.001$) and moderately significant on 5-7 days ($p<0.01$) after the treatment. In these mice, percentage of blood T lymphocytes to cells in lymphocyte rich fractions were also markedly reduced to 2.8%-36% of those of respective saline-treated controls through the entire period tested. Percentages of B lymphocytes in the anti-TG-treated mice were reduced. In contrast, similar administration of rabbit IgG had no significant effects either on total leukocyte, T or on B lymphocyte counts through the entire period tested.

Table 1. Effect of cyclophosphamide (CY) on total leukocyte counts and onpercentages of T and B lymphocytes*

Treatment	Days after treatment	Sex	Number of mice used	Cells		
				Total leukocytes ($x10^3/mm^3$)	T cell (%)	B cell (%)
Saline		Male	10	7.97±2.35	53.8±10.9	27.9±3.3
		Female	8	7.81±4.68	57.8±11.2	20.3±4.8
CY (300 μg/g)	2	Male	6	2.32±0.71†	60.1±21.5	12.5±3.1
		Female	5	3.15±0.58†	70.9± 8.2	15.4±2.9
	7	Male	7	5.01±2.31††	54.7±11.5	10.4±4.5
		Female	5	7.01±2.07	60.3±12.1	17.3±3.2
	14	Male	6	8.35±2.71	58.7± 6.2	15.4±1.3
		Female	5	8.07±3.23	54.9± 6.3	15.7±4.4
CY (400 μg/g)	2	Male	5	0.64±0.21†	72.6±17.5	8.5±2.1
		Female	6	0.73±0.41†	75.3±10.9	9.4±1.8
	7	Male	6	3.45±1.50†	67.2± 8.5	3.4±1.1
		Female	7	4.42±1.66†	68.3± 7.4	5.5±1.2
	14	Male	6	6.95±1.72	58.1± 8.3	6.4±2.4
		Female	6	7.40±3.08	63.2± 9.2	10.1±2.6

*Each BALB/c mouse was given a single intraperitoneal injection of CY (300 μg/g or 400 μg/g body weight). Mice were sacrificed and their white blood cells, T and B cells were counted on days indicated. Values are represented as the mean ± one standard deviation. Significantly deviated with $P<0.001$(†) and $P<0.01$(††) from those of respective saline-treated controls.

Table 2. Effect of anti-thymocyte-globulin (anti-TG) on total leukocyte counts and on percentages of T and B lymphocytes*

Treatment	Days after treatment	Sex	Number of mice used	Cells		
				Total leukocytes ($x10^3/mm^3$)	T cell (%)	B cell (%)
Saline		Male	12	7.82±2.96	56.7±7.7	26.5±9.2
		Female	10	7.95±1.51	58.6±9.8	21.5±6.8
Anti-TG	1	Male	4	0.97±0.31†	4.62±1.1	13.0±1.5
		Female	4	0.66±0.21†	1.63±0.1	16.3±4.8
	3	Male	5	2.44±1.12†	6.63±2.8	30.4±4.8
		Female	5	2.12±0.45†	6.67±1.1	29.3±1.4
	5	Male	4	8.55±0.28	9.31±0.7	27.2±1.6
		Female	5	5.19±1.63††	8.82±1.7	21.4±3.5
	7	Male	4	5.69±1.91	9.75±4.9	26.6±4.9
		Female	6	5.28±1.80††	10.2 ±4.0	19.7±7.2
	10	Male	4	8.02±1.27	18.3 ±4.6	29.3±5.8
		Female	4	7.06±1.47	21.2 ±8.7	27.1±5.6
Rabbit IgG	3	Male	7	7.65±2.47	58.3 ±5.8	23.3±5.8
		Female	6	7.42±1.98	63.1 ±7.4	17.4±3.3
	7	Male	8	7.23±2.51	62.5 ±7.0	28.4±7.1
		Female	6	7.82±2.70	54.0 ±6.2	22.4±4.3
	10	Male	5	6.98±1.65	60.3 ±9.8	27.8±4.8
		Female	5	8.03±3.24	53.8 ±5.5	28.9±6.3

*Each BALB/c mouse was given a sigle intraperitoneal injection of anti-TG or mabbit IgG. Micewere sacrificed and their white blood cells, T and B cells were measured on days indicated. Values are represented as the mean ± one standard deviation. Significantly deviated with $P<0.001$(†) and $P<0.01$(††) from those of respective saline-treated controls.

DISCUSSION

In this study, we have artificially regulated B and T cell functions in mice by *in vivo* administration of CY and/or anti-TG, and immunochemically measured their serum C1q and C3 levels in parallel with those of serum IgG.

Dosages of CY used in this study were 300 μg/g and 400 μg/g body weight and had strong leukopenic effects in BALB/c mice. Its suppressive effect on numbers of peripheral blood lymphocytes was more severe and long-lasting on the B cell compartment than on the T cell compartment, and the effect on B cells lasted at least up to 14 days after the administration (Table 1). These results actually agree well with the report by Stockman et al[27]. All the mice given the 400 μg/g dose in this study survived up to 14 days after treatment, which showed BALB/c mice were more resistant to the toxic effects of CY than (C57BL/6 xA) F1 mice[27]. A slight reduction of B cell populations besides a more severe reduction of T cell populations was observed in this study by using a commercially available anti-TG reagent (Table 2); the reason for which could not be identified, but it is most likely due to the low specificity of this antibody preparation as anti-mouse-Thy-1 reagents.

Treatment of the mice with CY diminished serum C1q levels as well as IgG levels (Fig. 1). Treatment with anti-TG reagent also diminished serum C1q levels (Fig. 2). These results and the fact that B cell population was also impaired in anti-TG treated mice as in CY-treated mice suggest that B cell impairment may cause reduced maturation of serum C1q levels as well as

that of serum IgG levels. These results may correspond to those obtained in patients with Bruton type hypogammaglobulinemia[5),6)], in which only their immunoglobulin producing system was impaired but their cell-mediated immunity was not apparently defective and both of their seum C1q and IgG levels were markedly diminished. These results also agree well with our previous report[28)] that embryonic surgical bursectomy of chicken diminished markedly their serum C1q as well as IgG levels.

Marked decreases of C1q were also reported in patients with Swiss type lymphopenic aggammaglobulinemia, in which both types of cell-mediated and huroral immunity were impaired, by Gewurz et al[29)], and in athymic BALB/c-nu (nude) mice by us[7)]. These reports have yielded a possibility, while it remains to bo further clarified, that decrease of serum C1q levels observed in the present study might be related to impairment of T cell populations by CY and/or asti-TG. In addition, because the lack of any effects of rabbit IgG either on blood lymphocyte populations (Table 2), serum C1q or C3 levels (Fig. 2), these regulatory activites of anti-TG are supposed to be immunologically specific.

Data presented in this report also showed that serum C3 levels were markedly increased in the mice treated with CY shortly after administration (Fig. 1), which might be caused by a possible enhancing effect of CY similar to that described in delayed-type hypersensitivity with a smaller dose of CY by Askenase et al[30)]. Mechanisms of such a conspicuous increase of IgG levels at 7 days after the anti-TG-treatment in male mice (Fig. 2) could not be clarified; no antibodies to rabbit IgG were detected by using a passive haemaglutination test with rabbit IgG -coated sheep erythrocytes (data not shown). Furthermore, data in this study showed that male mice were more susceptible to the suppressive effects of CY on serum C1q and IgG levels than female mice (Fig. 1). The exact mechanisms of this finding should be elucidated.

In conclusion, the present study indicates that there are some regulatory links between serum C1q levels and functions of B lymphocytes (and also possibly those of T lymphocytes) in mice.

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